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No. 27.

A MOLECULAR CONSTANT FOR SOURED MILKS.

III.—Very Old Samples.

By J. J. RYAN,
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[Read June 27. Published July 25, 1944.]

In previous numbers of these proceedings (1), (2), (3), molecular constants suitable for the detection of watering have been worked out for fresh milk, for milk held for about three weeks without preservative, and for milk preserved with potassium dichromate and held for about two months.

The further problem of deciding the genuineness or otherwise of samples of unpreserved milk, which have been held for several months, is often met with. The present and final note deals briefly with the possibility of extending the method of molecular constants to such old samples. For this investigation the original freezing point of the milk has been compared with a cryoscopic constant derived from analytical determinations of the lactose, chloride, and total phosphate, in the soured milk. The convenient approximate determination of lactose by refractometry employed in earlier articles is unsuitable for such old milks. Instead, lactose is taken as the difference between the total solids determined by the method of the British Government Laboratory (4) and the sum of the fat, protein, and ash. Chloride is determined by the method outlined by Ryan and Pyne (1), and soluble phosphate by the method of Pyne (5), as modified for sour milk (2). End point difficulties, sometimes met with in the last estimation owing to the sour milk on neutralization becoming yellowish or brown, can be overcome by adding a few drops of potassium chromate solution to the magenta standard. Alternatively, both chloride and phosphate estimations can be made on the sour milk filtrate or the centrifugally separated serum.

For a milk containing L grams lactose, C grams of chloride (as NaCl), and P grams soluble phosphate (as P_2O_5) per 100 grams of water:—Freezing point $\triangle = 0{\cdot}052\,L + 0{\cdot}615\,C + 0{\cdot}585\,P + a = K + a$, where a is the contribution of minor constituents and K the cryoscopic constant used here (1).

For the calculation of K the three percentages, lactose, chloride, and phosphate, must of course be corrected for the dry matter of the milk, and expressed as weights per 100 grams of water.

For nine samples of individual milks examined after holding for about three months at ordinary temperatures the values found for K varied from 0·443 to 0·466. When each value was compared with the original freezing

point of the corresponding milk determined by the Hortvet Cryoscope it was found that the sum of the lactose, chloride, and phosphate contributions furnished from 79·6 to 85 per cent., or on an overage 82·4 per cent., of the total depression (the real ratio is somewhat higher, as the formula for K is based on corrected freezing point depressions while the Hortvet values are uncorrected for supercooling, and average about 3 per cent. too high.)

Taking the average ratio obtained here, 82·4 per cent., and calculating on this basis the freezing points of the milks from the K values, figures are obtained which run from at most 0·018° C. higher to 0·019° C. lower than the actual freezing point as determined by the Hortvet.

H. J. Evans (6) has described a method of determining the original freezing point of soured milk which bears some resemblance to the present one. He dissolves the amount of lactose in the fresh milk (determined by the method of the British Government Laboratory) in the appropriate volume of a solution prepared from the ash in such a way that the calcium and phosphorus are thought to exist in a similar condition (as far as their effect on the freezing point is concerned) to that of the original milk. The freezing point of the artificial solution is then determined. The values thus obtained were found to differ from the original freezing points by amounts ranging from + 0·008° C. to − 0·019° C., differences comparable with these obtained here, using the molecular constant.

For the latter the maximum departure of the calculated freezing points from the values experimentally obtained on the fresh milks is about 3 per cent. By the use of either the molecular constant K or, what amounts to the same thing, the freezing point calculated from it, it should be possible therefore to estimate added water in very old samples of milk with an accuracy of about 3 per cent. This is probably as high a degree of accuracy as can be expected, taking into account the difficulties attending the determination of the original lactose, much of which will usually have undergone considerable fermentation by the time the analysis is made.

SUMMARY.

A molecular constant for old soured samples of milk, based on corrected lactose, chloride, and phosphate allows of the determination of added water with an accuracy of about 3 per cent.

REFERENCES.

(1) RYAN and PYNE (1934).—Sci. Proc. Roy. Dub. Soc., **21** (N.S.), 113.
(2) PYNE and RYAN (1937).—Sci. Proc. Roy. Dublin Soc., **21** (N.S.), 581.
(3) RYAN and PYNE (1940).—Sci. Proc. Roy. Dub. Soc., **22** (N.S.), 283.
(4) Somerset House Process—Reported by Richmond, Eldson and Walker, Dairy Chemistry, 4th Ed., 353.
(5) PYNE (1935).—Sci. Proc. Roy. Dub. Soc., **21** (N.S.), 223.
(6) H. J. EVANS (1936).—The Analyst, **61**, 666.

No. 28.

OBSERVATIONS ON A SEVERE STRAIN OF POTATO VIRUS X.

By PHYLLIS E. M. CLINCH,
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(Plates 15–17.)

[Read February 22. Published separately August 11, 1944.]

The potato virus now generally referred to as X (*Solanum Virus* 1, *Marmor dubium*), is probably the most widespread of those affecting potato stocks. In America it was found to be a universal constituent of the principal commercial varieties; and while many European varieties are at least partly free from X, others such as Up-to-Date and Duke of York are almost invariably infected.

It has been recognised for several years that different strains of X are in existence. Johnson (17) and Koch (19) distinguished between the "mottle" and "ringspot" forms of latent mosaic, the names being descriptive of the type of symptom produced on inoculation to tobacco. Köhler (23) made a similar distinction in the case of X viruses occurring in field potatoes in Germany, adding that each form contained strains of varying virulence. Murphy and McKay (26) observed that a latent top-necrosis virus was widespread in American material examined by them, and they equated it with the top-necrosis virus commonly carried by Up-to-Date (virus B). Recently, Clinch (9) showed that the Up-to-Date virus is a strain of X, readily distinguishable from other strains by its reactions in certain varieties of potato. Salaman (29) recognises six different strains of virus X. These vary from an extremely mild form X^H, which is almost symptomless in potato, *Datura stramonium*, and tobacco, to the strains designated X^S and X^N, which cause severe symptoms in these hosts. Salaman showed that these various strains of X were mutually protective.

In this country, the commonest form of X disease in commercial seed potato stocks is one causing a mild or almost imperceptible mottling of the foliage of affected plants. Murphy and McKay (26) used the term simple mosaic in reference to this disease, which they distinguished from other potato mosaic diseases by its effects on different varieties of potato. Clinch and Loughnane (10) observed that the symptoms evoked in tobacco and other solanaceous hosts by inoculation of simple mosaic from different potato sources were not identical. The form which they referred to as mosaic *ex* C produced in tobacco a definite pattern of chlorotic rings and necrotic spots, while the symptoms of the standard simple mosaic from the laboratory stock were more nebulous although of similar type. Similarly, in other solanaceous hosts the

two differed, but only in the intensity of the symptoms produced, except in intolerant varieties of potato such as Arran Crest and Epicure, in which the reactions are identical. Both would now be defined as mild forms of X. Koch and Johnson (20), who afterwards examined the same material, identified the milder form as "mottle" and the stronger one as "mottle and ringspot," a diagnosis which will be discussed later.

Because of the prevalence of simple mosaic, it had come to be more or less accepted that severe mosaic in the field was invariably due to mixtures of simple mosaic with other viruses, such as A and Y. However, it is now recognised that a strain of X exists in this country which, alone, is capable of causing a severe disease in potatoes. It was first encountered by the writer in 1934 in a single plant of a small crop of British Queen potatoes grown from certified seed in Co. Dublin. The writer's colleague, Dr. R. McKay, now believes that the same disease was present in crops inspected by him several years previously, and that it was probably diagnosed as crinkle at that time. In 1938 another colleague, Mr. J. B. Loughnane, observed it in seed potatoes stocks in Co. Donegal, and samples were also received from the Midlands and other centres. Mr. Loughnane and Mr. P. Keenan, Inspector in the Department of Agriculture, reported that only a very small percentage of infected plants occurred in any one crop, and that in some cases the plants appeared to be only partially infected. It was at first believed that the disease was a composite one, but inoculation studies failed to reveal an underlying virus other than X.

The present paper is an account of the symptoms and behaviour of this virulent strain. In order to avoid complications the same virus stock was used for all the reactions about to be described, fresh inoculations to virus-free potatoes being carried out each year.

Symptoms of Severe X in Potato Varieties.

The symptoms produced in 32 varieties of potato have been observed both in house-grown and field plants. Infection has been conveyed by sap inoculation, top-graft, and tuber core-graft, and is readily effected irrespective of the technique employed. Excluding Arran Crest, Epicure, and King Edward, which react with top-necrosis, all the varieties tested react in a more or less similar manner. The following is an account of the typical reaction produced in young, actively growing virus-free plants following sap inoculation by the rubbing method:—

Local necrotic lesions appear on the inoculated leaves after 6–10 days; these are circular, blackish, and small at first, but increase in size very rapidly and coalesce, so that large areas of the leaves wilt and turn grey. Eventually, these leaves wither and drop off. About 10–14 days after the appearance of local lesions systemic symptoms begin and develop with great rapidity. In the sub-apical leaves the veins clear, and pale blotches may appear, spreading

outwards from the main veins; then, suddenly, all the subsidiary veins become necrotic, black streaks appear on the undersides of the large veins, and the leaves collapse and wither in a short time. Superficial brown streaks may also occur on the stems and petioles. Thus the initial phase of the systemic disease is a distinctly necrotic one, and the leaves affected at the onset of secondary symptoms either drop off at once or dry out and remain hanging for a time (Fig. 1). The foliage which develops subsequently shows conspicuous symptoms of a different type. The leaves are severely puckered and rugose, and display a brilliant mosaic mottling as well as numerous dry, interveinal necrotic lesions. They are extremely brittle, and attain little more than half the normal size (Fig. 2). As a consequence of the stunting, chlorosis, and severity of the symptoms generally, a variety may be rendered almost unrecognisable in the field. The tubers appear normal, but the yield is considerably reduced.

During the phase of veinal streaking, especially when the latter is aggravated by dry conditions, the symptom picture resembles that of primary Y infection, but the subsequent mosaic mottling, which persists during the life of the plant, clearly distinguishes virus X. (The upper leaves of Y-infected plants are rugose, with a characteristic sheen but without a distinctive mosaic pattern). Even more readily might the secondary mottle symptoms of severe X infection be confused with the disease known as crinkle, due to X + A (25). Excluding the leaf drop and more widespread necrosis, which in the first year of infection serve to distinguish severe X from crinkle, the symptoms of the two diseases are very similar, those of severe X being the more conspicuous in Arran Banner, Majestic, and most other varieties.

In the second year of infection the leaf-drop symptom is absent. Otherwise the plants appear as in the first year of infection. Growth is stunted, the plants being about half the normal size; the leaves are severely puckered, and display a brilliant mosaic mottling together with numerous "rusty" necrotic lesions in the interveinal areas (Fig. 6).

The following varieties react in the manner described above:—Arran Banner, A. Cairn, A. Consul, A. Peak, A. Pilot, A. Signet, A. Victory, Ballydoon, British Queen, Catriona, Champion, Di Vernon, Doon Early, Doon Star, Dunbar Cavalier, Dunbar Rover, Dunbar Yeoman, Eclipse, Gladstone, Great Scot, Kerr's Pink, Majestic, President, Redskin, Shamrock, Sharpe's Express, Ulster Monarch.

Dunbar Standard was less severely affected than other varieties. The lower leaves did not drop, nor was the vigour of the plants so seriously impaired. The variety Irish Chieftain, which carries virus A, reacted with a severe crinkle ((25) (10)) accompanied by dropping of the lower leaves and interveinal necrosis.

The effect on the varieties Arran Crest, Epicure, and King Edward was exactly similar to that caused by milder strains of X. Following grafting, all three reacted with top-necrosis, and the "eyes" of the tubers were killed.

Sap inoculation resulted in local necrotic lesions, rarely followed by systemic necrosis and death of the plant.

Plants grown from tubers grafted with diseased cores showed systemic symptoms similar to those following sap inoculation (Fig. 2). Usually only one or two of the shoots showed infection on coming above ground, and sometimes all shoots appeared normal at first. Beginning in one or two shoots, the disease gradually spread to others until the entire plant was infected. The initial symptoms invariably took the form of a necrosis of all the fine veins in the sub-apical leaves. This led to leaf-drop accompanied by the mosaic symptoms, already described. If, as occasionally happened, a shoot had attained a height of 10–12 ins. before becoming infected, it failed to show the typical symptoms, the leaf drop and severe distortion being absent.

Introduction of Severe X to plants already infected with Mild X.

As expected, the previous presence in a potato plant of a mild strain of X completely prevented the introduction of the severe strain by sap inoculation. When the grafting method was employed, however, the severe strain was enabled to effect an entry. Following core-grafting of severe X into 9 President tubers infected with simple mosaic, only one plant showed the puckering and rusty necrosis symptomatic of severe X in some of its shoots. The plant was only slightly stunted as compared with control plants infected with severe X alone; and, with continued growth, the upper parts of the foliage showed only a mild mottle.

Three President plants infected with mild X were then top-grafted with severe X scions. After about 3 weeks the stocks showed, in addition to the mild mosaic mottle, some rather conspicuous yellow blotches with necrotic centres. Although the symptoms were not comparable in severity with those of control plants having severe X only, it was obvious that the severe strain had been introduced, but appeared to have difficulty in becoming established. On cutting the tops of the plants, however, the new shoots which developed showed more or less typical severe X symptoms, and when sap from them was inoculated to *Datura stramonium*, the full symptoms of severe X were produced. Six tubers from plants infected with the mixed strains were grown in the field in the following year. In most of the resultant plants the majority of the shoots showed typical severe X symptoms on coming above ground, the remaining shoots showing a mild mosaic; but, as the plants developed, the upper leaves of the severely infected shoots flattened out and showed only the mottle symptoms of the milder strain. Consequently, by the end of June, when the plants were approaching maximum size, they had quite a distinct appearance from plants in an adjoining drill which were infected with the severe strain only, and which exhibited fully severe symptoms throughout the season.

Symptoms in Solanaceous Hosts other than Potato.

Datura stramonium.—The inoculated leaves develop circular necrotic lesions after 4–6 days. These increase rapidly in size, and the leaves soon wither and drop off. Systemic symptoms appear in the upper leaves about the 9th day. The veins clear and rapidly become necrotic, so that large areas of the leaves (usually the distal portions) wilt and then dry out (Fig. 7). The remaining areas show ruffling and distortion and a brilliant mosaic pattern of chlorotic rings and blotches interspersed with numerous "rusty" brown necrotic lesions. Growth is severely checked with the onset of symptoms but continues later, the new leaves displaying conspicuous mosaic symptoms accompanied by a lesser amount of necrosis. The leaves generally are much reduced in size, and the plant, as a whole, severely stunted. The flowers are also affected, developing grey or brownish patches on the calyces and corollas, usually resulting in premature withering. The virus is not carried in the seed from infected plants.

Nicotiana glutinosa.—After 4 days the inoculated leaves display small circular grey spots which rapidly develop into dark zoned lesions about 5 mm. or more in diameter; these coalesce and dry out, whereupon the leaves wither and drop off. Meanwhile, about the 9th day, symptoms appear in the young leaves at the top of the plant. The veins turn yellow, then rapidly become necrotic, particularly in the distal portions of the leaves, which soon wilt and dry out. The remaining areas are ruffled and distorted, and display a brilliant yellow mosaic pattern interspersed with numerous rusty necrotic lesions, symptoms which continue to appear in all subsequent leaves. The plant is severely stunted, and the effects generally are extremely severe (Fig. 8).

Lycopersicum esculentum (Tomato).—The inoculated leaves develop necrotic rings or circular blackish lesions accompanied by other necrotic configurations associated with the veins. Secondary symptoms appear in the top of the plant 9–10 days after inoculation; the leaves curl downwards, the veins clear, then small greyish patches develop all through the interveinal areas. These spread rapidly and coalesce, whereupon the leaves collapse and wither. As new leaves unfold they display similar symptoms, except that the wilting is less severe. Interveinal necrosis remains the dominant feature of the symptom picture (Fig. 5), being sometimes preceded by a conspicuous yellow mosaic in the young growth. The lesions usually appear first in the terminal leaflet, then spread to the remaining leaflets. They may be circular or irregular in shape, and, according as growth is "hard" or "soft," they may be dry and reddish-brown in appearance or grey and water-soaked, respectively. In the latter case they spread more rapidly, and the leaves soon wither. No lesions were observed on the stems or fruit.

Tomato plants inoculated with a mixture of severe X and tobacco mosaic (*Nicotiana Virus* 1) were killed by a virulent necrotic disease within 14 days after inoculation. When milder strains of X (simple mosaic and mosaic *ex* C)

were substituted for the severe strain, the symptoms were correspondingly milder, consisting of more or less extensive necrotic lesions on the leaves and petioles, and streaking of the main stem also in the case of mosaic *ex* C. The actual growing points, however, survived.

Solanum nodiflorum.—Local lesions are numerous and consist of purple-fringed red rings with chlorotic centres; the inoculated leaves rapidly turn yellow and drop off. Secondary symptoms appear in the top leaves after 10 days; the veins develop a conspicuous yellow colour, and their development appears to be inhibited. The interveinal areas are ruffled and distorted, and are covered by dark reddish markings, many of which are ring-shaped. The necrotic lesions enclosed by them turn yellow, and the leaves drop. Within a few weeks only a few leaves at the top of the plant remain. These are puckered and distorted, and display a conspicuous yellowish green mottle accompanied by dry necrotic lesions. The plant as a whole is severely stunted.

Solanum nigrum.—Reacts very similarly to *S. nodiflorum.* Primary symptoms take the form of circular black lesions which increase rapidly in size; the intervening tissue turns yellow, and the leaves drop off. Secondary symptoms develop in the top leaves, the veins becoming yellow at first and then almost necrotic; the interveinal areas show puckering and severe chlorosis together with dry necrotic lesions. Affected leaves fall at the slightest touch, and eventually only a few leaves at the top of the plant remain. Many of the blossoms drop prematurely, and the plant as a whole is severely stunted.

Capsicum annuum.—The inoculated leaves develop severe zoned necrotic lesions, and then drop off. Systemic symptoms appear first in the top leaves, the veins of which become severely chlorotic; numerous necrotic rings and angular markings develop in the interveinal areas, and the tops of young plants may be killed. If the tops survive the leaves are puckered and distorted, and show pronounced mottling accompanied by irregularly shaped necrotic lesions.

Nicotiana tabacum (Tobacco) *var.* White Burley.—About 4 days after infection the inoculated leaves show circular pale yellow areas, which tend to develop necrotic outlines. Systemic symptoms appear in the upper leaves about 10 days after inoculation and take the form of a conspicuous mosaic, the leaves being covered by innumerable small chlorotic rings and circular areas, usually outlined by bands of reddish or necrotic tissue or having a central necrotic spot. As new leaves develop they in turn display a conspicuous mosaic, which begins as a yellow spotting at the tip of each newly unfolded leaf. The growth of the plant as a whole is stunted. The severe strain was recovered unchanged from White Burley plants which had been held in the greenhouse for 6 months (October–April) after inoculation.

Nicotiana tabacum (Tobacco) *var.* Orinoco.—Circular blackish necrotic lesions develop on the inoculated leaves after 3–5 days; these increase rapidly in size, and coalesce, and in a short time the leaves wither and fall off.

Usually there are no further symptoms in this variety, but secondary symptoms occasionally develop. Vein clearing in the top leaves may be followed by the development of chlorotic rings and spots which rapidly become necrotic; but more often the secondary symptoms are exclusively necrotic, consisting of necrotic rings, circular spots, and streaks, all of which are associated originally with the veins. The rings increase in size or become surrounded by larger rings which may coalesce, so that large areas of the leaf wither. The disease advances acropetally, but the necrotic reaction in the leaves apparently serves to hinder the multiplication of the virus, so that invariably the upper portion of the flowering shoot appears healthy and may in fact be virus-free. In the plant shown in Fig. 9, photographed seven weeks after inoculation, virus X could not be demonstrated in the three healthy appearing leaves immediately above the diseased ones, nor in the symptomless half of the uppermost diseased leaf. All these areas responded with local necrotic lesions to severe X inoculum, besides which discs of tissue previously removed from them failed to produce symptoms on inoculation to *Datura*. When the tip of the plant was cut off, three axillary shoots which developed immediately below the point of severance appeared healthy; and when removed and rooted separately they produced virus-free plants. In the meantime, the parent plant produced further axillary shoots on the lower part of the stem, which in course of time showed necrotic symptoms once more.

Non-solanaceous Hosts of Virus X.

Hosts of virus X outside the family *Solanaceae* are almost unknown. Jones *et al.* (18) recorded *Amaranthus retroflexus* L. as a host of latent mosaic. Salaman (29) experimented with plants of twelve different families and recorded systemic infection only in two, *viz.* chrysanthemum and *Browallia speciosa*. The latter he classifies as a member of the *Scrophulariaceae*, but Willis (32) and other authorities include this exotic species in the *Solanaceae*. Of the remaining plants a small number, including red beet and sugar beet (*Chenopodiaceae*) and horse beans (*Leguminosae*), reacted with local necrotic lesions.

In the present work a number of common weeds from the potato fields and surrounding hedges at Glasnevin were inoculated with severe X sap. The results obtained were as follows:—

Lamium hybridum (*Labiatae*).—This host becomes infected fairly readily, although the percentage of successful infections is not so high as in solanaceous hosts. After about 10–14 days, pale-yellow or light-brown lesions usually appear on the inoculated leaves. They may be more or less rounded at first, but later the outlines become diffuse and they develop into irregularly-shaped dark rusty patches. Systemic symptoms consist of a diffuse mottling accompanied by light-brown or reddish-rusty lesions, the latter occurring

mainly on the intermediate leaves of the plant. Growth is stunted and the infected plants have a sickly appearance. The symptoms produced by simple mosaic (10) were of the same type but distinctly milder. Both strains were recovered unchanged on *Datura* and *N. glutinosa*. This appears to be the first record of systemic X infection of a non-solanaceous weed native to these islands.

Veronica agrestis (*Scrophulariaceae*).—Circular, black local lesions began to appear after 10–12 days, and reached a diameter of about 4 mm. There was no systemic infection.

Beetroot and Mangold (*Chenopodiaceae*).—Six days after inoculation small circular lesions appear all over the rubbed leaves. These coalesce and the affected leaves wither and drop; there are no systemic symptoms. Simple mosaic induces similar lesions, but they are fewer in number and slower in developing.

No infection was secured in the following plants: Caryophyllaceae: *Stellaria media*. Chenopodiaceae: *Chenopodium album*. Compositae: *Carduus arvensis, Chrysanthemum indicum, Senecio vulgaris, Sonchus oleraceous, Taraxicum officinale, Tussilago farfara*. Convolvulaceae: *Convolvulus arvensis*. Cruciferae: *Brassica oleracea, Capsella bursa pastoris*. Fumariaceae: *Fumaria officinalis*. Labiatae: *Galeopsis tetrahit, Salvia splendens, Stachys sylvatica*. Papavaraceae: *Papaver rhoeas*. Plantaginaceae: *Plantago lanceolate*. Polygonaceae: *Polygonum aviculare, Rumex obtusifolius*. Ranunculaceae: *Ranunculus repens*. Rosaceae: *Potentilla anserina*. Rubiaceae: *Galium apperine*. Scrophulariaceae: *Antirrhinum majus*.

Physical Properties of Severe X.

Results of repeated tests with freshly-expressed sap showed that the severe strain was similar in its physical properties to the common mild strains of X. These results were as follows:—

Thermal inactivation point.—68° C. (10 mins. exposure).

Filterability.—Sap was highly infective after passage through L 1, L 3, and L 5 grades of Pasteur-Chamberland filter candles.

Longevity in vitro.—At 17°–20° C. there was a progressive decrease in infectivity of crude sap up to approximately 28 days, when inactivation was usually complete. Crude sap held at 5° C. was still infective after 60 days; the period necessary for complete inactivation at this temperature was not determined.

Resistance to dilution.—Infection was obtained at a dilution of 1 in 50,000 but not at 1 in 100,000.

Serological Experiments.

In the course of some work on the serological reactions of potato viruses, tests were made both with the severe and mild (mosaic *ex* C) strains of virus X,

the results of which have a bearing on the present subject. The immune sera were obtained from rabbits, and the immunising antigens were the freshly extracted juices of potato foliage diluted 1 in 5 with water and clarified by filtration. The injections, six in number, were given intravenously at 3–4 day intervals, the doses being 0·5, 1·0, 1·5, 2·0, 2·0, 2·0 c.cs. Ten days after the last injection the animals were bled, and the serum obtained was centrifuged and stored in the frozen state. To prepare the test antigens expressed juice was diluted slightly, then twice frozen overnight, thawed, and centrifuged, when a clear light-brown supernatant liquid was obtained.

In carrying out flocculation tests, 2 c.c. of antigen was mixed with 1 c.c. of serum, serial dilutions being made in 0·85 per cent. saline. The mixtures were incubated at 37° C. for 30 min., then held at 5° C. overnight to allow precipitates to settle. Healthy plant sap was tested at the same time as virus extract with the usual controls of sap and saline and sap and normal serum. The sera were not absorbed beforehand, as precipitates due to healthy potato proteins and their antibodies were slight; they did not occur at all when the test antigens were prepared from *Datura stramonium*.

The results of the tests showed that both strains of X induced an anti-serum of high titre following a single course of injections as described above; in fact, blood probes indicated that active serum could be obtained even after the 4th injection. The high serological activity of virus X has already been demonstrated by Chester (6), Spooner and Bawden (31), and others. Incidentally, it may be mentioned that the writer failed consistently to obtain a serum active towards potato virus A, even after three courses of injections, while the reactions obtained with anti-Y sera were weak and unsatisfactory (cf. Gratia and Manil (15) and Hansen (16)). This applies to tests with green saps according to Chester's (8) method, as well as to those with cleared antigens.

In these experiments no significant difference was detected in the comparative ability of the severe and mild strains of X to induce antibody formation. The sera of both reacted at approximately the same dilutions; if anything, the anti-severe X sera were slightly more active than the anti-mild X sera; but considering the method of preparation of the antigens and the probable differences in reaction of individual rabbits, no distinction could be made between the two strains in this respect.

Several tests were made with anti-X sera and extracts of X-infected and healthy tubers. The tissues were macerated with a little distilled water, and allowed to stand for 3 hours at room temperature. The juice was then filtered through muslin, centrifuged, placed in a cold chamber (5° C.) overnight, and again centrifuged. Separate extracts of the skin and flesh of the tubers and of areas surrounding and including the dormant buds, were prepared; but contrary to the results of Gratia and Manil (15), none of these extracts flocculated with immune serum. Extracts of diseased roots which were prepared in the same manner showed a reaction, but this was much weaker than that

obtained with leaf extracts. One concludes from these results that the virus is less concentrated in the roots than in the leaves, and that the quantity present in dormant tubers is extremely small. Confirming the latter view is the fact that only a very low percentage of infection was obtained in inoculation experiments with the juice from infected tubers. Other workers, e.g. Burnett and Jones (5) and Johnson (17) experienced the same difficulty in securing infection with tuber extracts.

Attenuation of the Severe X disease in Potato.

It has been mentioned that the first sample of severe X examined by the writer occurred in a crop of British Queen plants grown in Co. Dublin. Inoculations were made from the diseased plant to virus-free British Queen, and the stock thus infected was maintained in the usual way by tuber propagation in the glasshouse. In the 3rd year of infection an attenuation in symptoms was observed, which persisted in the following year. In the 5th year of infection all traces of the virulent disease seemed to have disappeared from the stock, which, when grafted on ten other varieties of potato, produced only a mild disease of the simple mosaic ((26) (10)) type.

In view of this behaviour a careful examination was made of the reactions of different varieties of potato to severe X over a period of 3–4 years. The virus used was isolated from field plants in Co. Donegal, and appeared to be identical in every respect with that isolated in Co. Dublin. Certain varieties were inoculated in the glasshouse, and propagated subsequently both in the glasshouse and in the field. These included Arran Banner, Arran Cairn, Arran Consul, Arran Signet, Ballydoon, Champion, Di Vernon, Doon Early, Kerr's Pink, and President. Other varieties were inoculated in the field, and the infected stocks kept under observation for four successive generations. These included Arran Peak, Arran Pilot, Arran Victory, British Queen, Catriona, Dunbar Standard, Dunbar Yeoman, Great Scot, Gladstone, Majestic, Redskin, Shamrock, and Ulster Monarch. The course of the disease was essentially similar in all cases. In the first year of infection the plants showed severe mosaic, puckering and necrosis of the foliage, and leaf drop; in the 2nd year similar symptoms but without the leaf drop. During this period, inoculation to other solanaceous hosts resulted in the typical severe X symptoms. In the 3rd year of infection an appreciable change took place. The plants, on coming above ground, showed the fully severe symptoms of the previous year, but as growth continued the new leaves appeared free from puckering and necroses, and exhibited only a relatively mild mosaic mottle (Fig. 3). The symptoms produced on *Datura* by inoculation from the upper and lower leaves of such plants were quite distinct; in the latter case conspicuous local necrotic lesions were followed by the systemic symptoms typical of the severe strain; in the former case local lesions were absent or inconspicuous, stunting was

negligible, and the mosaic symptoms corresponded to those of an X virus of mild or medium intensity (Fig. 7). Sometimes the merging of the severe disease into the mild form was marked by transient intermediate symptoms of conspicuous yellow blotches on otherwise green leaves. Inoculation to *Datura* showed that the severe strain was present in the chlorotic areas, a mixture of the severe and mild strains in the tissues immediately adjoining them, while elsewhere only the mild strain was present.

By the 4th year of infection the potato plants were entirely free from the puckering and necrosis characteristic of the severe strain. Symptoms were reduced to a yellowish blotchy mottle on the lower leaves, and even this was extremely mild in some units. The degree of stunting was negligible, and a striking contrast was afforded by the appearance of two plants, one in the 2nd and the other in the 4th year of infection growing side by side in the field (Fig. 6). So far as could be judged from mass inoculation to *Datura*, the foliage of the recovered plants was free from the severe strain and contained only a virus of the common mild X type. However, as separate inoculations from finely dissected portions of the mottled leaves were not undertaken, the possible existence of the severe strain in minute areas of these leaves cannot be ruled out.

This reduction in virulence occurred consistently irrespective of variety or whether the plants were grown in the glasshouse or in the field. Although the transformation usually occurred in the manner described above some variations in this procedure were observed. For example, one plant of Kerr's Pink grown in the glasshouse showed attenuation of symptoms in one shoot in the 2nd year of infection, while the remaining shoots were fully severe throughout that season (Fig. 4). Of six plants grown from tubers of this unit in the following year four were infected with severe mosaic and two with a milder mosaic, and inoculations from them to *Datura* resulted in severe and mild symptoms, respectively. Eventually all units of the clone recovered. This phenomenon of premature recovery in isolated shoots was also observed in field grown plants of Kerr's Pink and other varieties. Occasionally a batch of plants showed general recovery in the tops of the shoots in the 2nd year of infection; in such cases a mild strain or, more often, a mixture of the severe and mild strains of X could be isolated from the apical growth; the succeeding generations of such plants generally showed the intermediate yellow blotch symptoms, already described, in their lower leaves. On the other hand, there were instances of individual plants of certain clones failing to show initial symptoms of recovery until the end of the 4th year of infection, although the remainder of the stocks showed attenuation in the previous season. Generally speaking, however, the transformation from the severe to the mild X disease was complete in the 4th year of infection.

It is possible that different environmental conditions may hasten or retard the diminution in virulence of the severe X disease. This view is

suggested by the fact that a group of field plants which showed milder symptoms as early as the 2nd year of infection had been subjected to warm, dry weather during the period of most active growth. Furthermore, it has been observed that the effect of bright sunlight is to cause a lessening in intensity of the mosaic symptoms, although the fact that severe symptoms develop with the advent of duller conditions indicates that this is mainly (but perhaps not entirely) a masking effect. While it cannot be stated with certainty that warm dry conditions facilitate the transformation from the severe to the mild disease, there is a certain amount of evidence pointing in this direction. On the other hand, the phenomenon of premature recovery in isolated shoots suggests control by internal, rather than external, factors. So far no evidence has been obtained that the subjection of infected tubers to high or low temperatures affects the virulence of the disease symptoms in the subsequent plants; and in fact the alteration seems invariably to occur in the foliage during the growing season. It was observed that the enforced production of new growth at the transitional period served to extend the period of predominance of the severe strain. Thus, on cutting back in the 3rd year of infection a young President plant whose lower leaves showed the virulent disease, the axillary shoots which were stimulated to growth showed the full symptoms of the severe strain, and continued to do so almost to the end of the season. Corresponding plants which had been allowed to grow naturally produced mildly infected foliage throughout the same period, so that the amount of severely diseased foliage was much greater on the cut than on the uncut plants at the end of the season.

Although certain factors may possibly affect the rate of transformation from the severe to the mild disease, the occurrence of the change seems to be inevitable; and having once attained the mild form the disease appears to become stabilized. So far no alteration in the reverse direction has been observed, and attempts to reproduce severe symptoms in fully recovered plants by cutting the tops or by growing them under different environmental conditions have been unsuccessful. It cannot be said that the strain of mild X which replaces the severe strain is identical in every plant or clone. The symptoms in the recovered potato plants vary, as already stated, from a clear blotchy mosaic to an almost imperceptible mottle; and such variations may be found between clones of the same variety infected at different times. There is a corresponding difference in the symptoms produced in *Datura* by inoculation from recovered plants, but this difference, both in potato and in *Datura,* is one of degree and not of kind. In general, the strains present in the recovered plants would correspond to the simple mosaic and mosaic *ex* C already mentioned. The severe strain may be said to differ in kind, as well as in degree, from such forms by reason of its characteristic symptoms of necrosis, distortion, and severe stunting.

EXPERIMENTS ON THE MOVEMENT OF SEVERE X IN U.S.D.A. SEEDLING 41956.

Numerous attempts to introduce the severe strain to 41956 by grafting or by sap inoculation with the aid of carborundum powder were unsuccessful. The 41956 plants failed to show symptoms, and were proved to be virus free by inoculation to *Datura* and by grafting on healthy Arran Crest and President, as well as on President containing mild X; no alteration of symptoms was effected in the latter. This result, besides confirming the statements of other investigators regarding the immunity of 41956 to X, also helped to dispose of an early idea that the severe disease might be due to a mixture of X with another virus, the latter being responsible for the necrotic symptoms.

It has been mentioned in a previous paper (9), however, that when 41956 was inserted as an intermediate scion in double-grafted plants, virus X was able to pass from an upper infected scion through the 41956 into a susceptible basal stock. The latter showed the typical X symptoms, but the 41956 remained normal in appearance, and attempts to demonstrate the presence of any virus in its foliage were unsuccessful. It appeared, therefore, that the virus had passed through the 41956 without multiplying therein. The time taken between the final grafting and the development of symptoms in the basal stocks of these double-grafted plants was of the same order as in single-grafted plants. Several experiments were carried out in this manner, all of which gave similar results.

Experiments were then made to determine whether virus X could travel upwards through 41956 with the same facility as downwards. Arran Banner plants were used as the basal stocks in two experiments. They were grafted, when 8–10 ins. high, with scions of 41956, which were allowed to grow at least 8 ins. in length before being top-grafted with scions of healthy Arran Banner. Control plants were similarly double-grafted, except that a healthy susceptible variety was substituted for 41956 as the intermediate scion. The plants were grown in 10 in. pots, and received liberal manuring during the season. Most of the top scions grew at least 10–12 ins. in length, and the intermediate scions and basal stocks were extremely vigorous.

In the first experiment the six Arran Banner basal stocks were originally healthy and were not inoculated with the severe X virus until the top scions were fully established; this was on June 9th, nearly 3 months after planting. After 14–18 days mosaic symptoms began to appear in the leaves of the basal stocks. Early in July the top scions and the shoots of the basal stocks were cut back to induce new growth. About 10 days later symptoms appeared in the intermediate scions of the controls, and after a further 7 days in the top scions of the same plants. Of the three plants having intermediate scions of 41956, however, none became infected with virus X in the upper scion up to the time of lifting on September 4th. The 41956 scions, therefore, had apparently served to prevent the passage of X from the basal stocks into the upper scions.

A second experiment was carried out in exactly the same manner except that the basal stocks were grown from X-infected tubers. The object of this was to save the incubation period following inoculation, and thus effect the introduction of the virus to the scions at an earlier stage. Both scions were established in from 2 to 2½ months after planting. Altogether 14 plants were grafted as described above, 10 with intermediate scions of 41956, and 4 controls with intermediate scions of healthy Arran Banner. The latter developed symptoms of severe X in 14–27 days after grafting, so that they were already systemically infected before the top scions were affixed; the top scions of these plants also showed symptoms in due course after they had become established. Of the 10 experimental plants with intermediate scions of 41956, the severe X reached the top scions in only two cases, although the plants were kept growing for 2 months after the final grafting. During the course of the experiment shoots were taken from the 41956 portions and grafted on Arran Crest potato, but even in those plants of which the top scions, as well as the basal stocks, were showing symptoms of severe X, the presence of the virus in the intermediate 41956 was not demonstrated.

In a third experiment 5 plants of President infected with a combination of viruses X and F (11) were grafted with 41956. In all cases the mild yellow spotting symptomatic of virus F appeared in the 41956 scions after 3–4 weeks. Top scions of healthy President were then affixed, two of which failed to become established. Of the three which grew actively, two became infected with virus F only, and the remaining one with both viruses.

These results show that the insertion of an intermediate scion of 41956 usually constitutes a barrier to the ascent of virus X in pot-grown potato plants over two months old. No such barrier is effected in similar plants by an intermediate scion of an X-susceptible variety; nor does 41956 affect the ascent of a virus to which it is susceptible, e.g. virus F. The ability to move upwards in such plants appears, therefore, to be bound up with the ability to multiply in the tissues to be traversed. Since movement of virus X through 41956 must be passive (being independent of multiplication) there is thus evidence of two types of movement of virus X in potato stems, the one passive and the other dependent upon multiplication.

In the case of the tobacco mosaic virus, which has been extensively studied in this connection, it has been established that the movement of the virus is intimately bound up with that of elaborated food materials, and the conclusion has been reached that the main path of rapid movement is in the phloem, through which the virus is transported mechanically in the food stream. A slow cell-to-cell movement is also envisaged, but differences of opinion exist regarding the actual mechanism involved in this process. It seems likely that this concept of the movement of tobacco mosaic virus is also applicable to virus X. There already have been indications of a correlation between the movement of X and that of elaborated sap (9), and the present results furnish

additional evidence of this relationship. In a plant such as the potato, which forms underground storage organs and does not normally set seed, the main flow of metabolites would be increasingly in a downward direction from the time tuber formation had been initiated. Cutting the tops of the plants with consequent production of new axillary shoots, would cause movement of food materials towards these new centres of utilization, but might not necessarily induce a complete reversal of flow if tuber formation were active. In the writer's experiments the passive movement of X, as exemplified by that occurring through 41956, took place freely and rapidly in a downward direction in pot plants which must have been in a state of active tuber formation, since they were about 2½ months old by the time union of scions was effected. Passive movement in an upward direction, however, occurred rarely in plants of this age, and failed altogether to take place in plants at a more advanced stage of growth. On the other hand, the type of movement which is apparently bound up with multiplication took place in an upward direction irrespective of the age of the plants, so that the direction of such movement can obviously be counter to the main flow of metabolites. A movement involving multiplication would presumably exclude the xylem vessels, if not the sieve tubes; hence the parenchymatous tissues are the most likely path of travel in this case. This movement is probably of the slow cell-to-cell type mentioned in regard to tobacco mosaic virus, although in the case of the latter virus it has not been shown that such movement is necessarily dependent upon multiplication.

The inability to demonstrate virus X in shoots from 41956 scions which the virus has obviously traversed may be due to a low concentration of virus in the scions. Bennett (4) has pointed out that the concentration of mosaic viruses in phloem is probably low, and it seems likely that virus X is confined to the phloem in the variety 41956. If the number of virus particles in the 41956 scion at any given time were small, the securing of infection by grafting shoots therefrom on healthy plants would obviously be fortuitous.

SPREAD OF SEVERE X IN THE FIELD.

It has not so far been shown that any insect vector is implicated in the spread of virus X in the field. Schultz and his co-workers (30) found that X-free potato seedlings contracted latent mosaic when grown in contact with infected plants or when brushed with diseased foliage; but the possibility of insect transmission could not be excluded in their experiments. Loughnane and Murphy (24) proved, however, that spread of X (simple mosaic) resulted from contact between the foliage of healthy and diseased plants grown in a glasshouse free at least from the larger sucking insects. They also showed that infection resulted from contact between the haulms of healthy and

X-infected plants in the field, although proportionately only about half as many plants (14 per cent.) contracted infection in the field as in the glasshouse. Infection was not contracted as a result of root contact only.

Experiments on the spread of severe X in the field were carried out during the three consecutive seasons 1940–42. The plants were grown in an isolated plot situated amongst beans or wheat and about 500 yds. away from other experimental potato plots. The location of the plot in the field was altered each year. The drills were 30 ins. apart and the plants 18 ins. apart in the drills. The plot was hand-worked and the chances of infection due to other than natural causes were reduced to a minimum. Majestic was selected as the principal indicator variety, and all the experimental plants used were derived from the same virus-free stock. Tests were also made with the intolerant varieties, Arran Crest and Epicure.

1940 Experiment. — The 1st drill in the plot contained 12 healthy Majestic plants, and the adjoining drill 12 plants of healthy Arran Crest. Next came a drill of Epicure, and then in the 4th drill 12 plants of Arran Banner infected with severe X. The first drill of Majestic was therefore situated at a distance of 7½ ft. from disease sources. Twelve more healthy Majestic plants were grown in contact with infected sources, the diseased and healthy plants being situated alternately in the drills. Twelve plants of healthy Arran Crest were also grown in contact with diseased plants in the same way. The remainder of the plot was occupied by plants of different varieties infected with severe X, excepting one drill in which were grown 12 plants of President infected with a mild strain of X. The latter was included for the purpose of observing whether any change occurred in the intensity of the mild disease.

The tubers were planted on 4th May. On 14th June, when the plants were 12–15 ins. high and growing vigorously, sap from each of the 24 Majestic plants was tested for X by inoculation to *Datura* and by means of precipitin tests with anti-X rabbit serum; the results were negative. They were again tested on 2nd July when the leaves of adjacent plants were first beginning to touch, but proved to be virus-free. By the middle of July there was generous contact between the haulms of adjacent plants, and a few weeks later the plants of adjacent drills were touching. On 9th August the Majestic plants were tested once more by inoculation from the upper leaves to *Datura* with negative results. On this data none of the test plants of Majestic, Arran Crest, or Epicure showed systemic symptoms of severe X, nor were any such symptoms observed up to the time the plants matured. The crop was lifted on 17th September, and the entire tuber yield of each test plant was saved, the units being stored separately.

In the following year all tubers were planted in the field, and examined carefully for disease symptoms. It was found that not a single plant derived from the 12 units of Majestic which were not in contact with severe X sources

had contracted infection; nor had any infection occurred in the units of Epicure and Arran Crest, irrespective of whether they were in contact with diseased plants or not. On the other hand 8 of the 12 Majestic plants which had been grown alternately in the drills with diseased plants had become infected—but in no case had the virus reached every tuber of the infected plant. On an average, 3 tubers out of the average total yield of 11 carried infection. Furthermore, in the majority of cases apparently healthy and diseased shoots came from the same tuber. The manner of appearance of the disease was similar to that occurring when tubers were infected by core graft, i.e. the initial symptoms consisted of blackish necroses on the leaves and streaking of the veins, the severe mosaic symptoms developing subsequently. It was quite common to find, in the middle of June, 4 shoots of a plant fully infected with severe X, the remaining shoots being apparently healthy; a month later the initial symptoms of veinal streaking and interveinal necrosis would have appeared in the previously healthy shoots, and eventually the entire plant would be affected. When initially healthy shoots became infected in this manner through spread of virus from other shoots on the same tuber, the fully developed basal leaves were the last to show infection, and appeared healthy for a considerable time after symptoms were visible in the tops of the shoots.

1941 Experiment.—The layout of this experiment was similar to that of 1940, but the number of test plants was increased. One drill of 15 healthy Majestic plants was grown at a distance of 7½ ft. from the sources of disease, while another drill was grown alongside a drill of infected plants. In addition, 15 Majestic plants were grown in the same drills as severe X plants, the diseased and healthy plants alternating in the drills. Fifteen plants each of the intolerant varieties Epicure and Arran Crest were also grown, being separated by a distance of one drill from diseased plants.

Owing to extremely dry soil conditions, the plants did not attain their usual size, growth was "hard," and the plants matured early. Consequently the degree of contact between plants of adjoining drills was less than in the previous year. The leaves of adjacent plants in the same drill began to touch about 8th July, and were in close contact by the end of July. During August a single shoot on each of 2 Majestic plants in contact with diseased plants was observed to show necrotic lesions and streaks on the leaves, and inoculation to *Datura* confirmed the presence of severe X in these shoots. Apart from these two cases, however, no symptoms were observed in any of the test plants of Majestic, Arran Crest, or Epicure. When the tubers of the experimental plants were grown in the following year it was found that only those Majestic plants which had been in contact with diseased sources in the drill had contracted infection, thus confirming the results of 1940 in every respect. The actual number of contact plants infected was 7 out of 15, i.e. about 47 per cent.

1942 Experiment.—In this experiment, which was similar to the preceding

ones, it was considered unnecessary to grow Majestic plants in contact with diseased sources, as the spread of virus between adjacent healthy and diseased plants in the same drill appeared to have been amply confirmed. Twenty-four healthy plants of Majestic were, therefore, grown at a distance of 5 ft. from a drill of diseased plants, while 12 healthy plants of Arran Crest were grown in contact with diseased plants. All these plants remained healthy in appearance throughout the growing season, and on 26th August the Majestic plants were tested for virus X, with negative results. When the tubers of the experimental plants were grown in the following year, it was found that not a single unit had become infected.

The conclusions to be drawn from these experiments are that in the case of a tolerant variety such as Majestic, severe X is transmitted freely by contact between adjoining healthy and diseased plants. Taking into consideration the results of Loughnane and Murphy (24) it may be assumed that transmission is effected through the leaves rather than through the roots of the plants. As no transmission whatever took place between diseased and healthy plants which were not actually touching, even though the distance separating them was at most 7½ ft., it may be concluded that under Glasnevin conditions insects are not operative in the spread of this virus. In the 3 years during which the experiments were in progress, thrips were abundant on the potato plants, being especially numerous in 1941. Aphids, although not plentiful, were present each year, and were sufficiently numerous to effect spread of leaf roll and virus Y in nearby plots on the same farm. Leaf hoppers were also observed on the potato plants in each of the three seasons. The results indicate, therefore, that these insects may be ruled out as possible vectors of severe X.

The absence of infection in the intolerant varieties Arran Crest and Epicure, even when grown in contact with diseased plants, is in accordance with the general experience that plants which react to a virus with top-necrosis do not contract infection with that virus in the field, due, it is believed, to the destruction of cells at the site of inoculation as a result of which movement of virus is restricted.

It is interesting to note that, although 67 per cent. of the "contact" Majestic plants actually contracted infection in 1940, none showed systemic disease symptoms in that year; and in 1941, when 47 per cent. infection occurred, close inspection revealed only the initial symptoms of the disease, and then only in a single shoot of each of two infected plants. In view of the severity of the reaction of Majestic to severe X, it is most unlikely that the virus was systemic in the foliage of those plants or shoots which remained healthy in appearance. All such foliage tested proved in fact to be virus-free. Hence the virus reached the tubers in the majority of cases without systemically infecting the foliage. In explanation of this it may be recalled that the leaves of adjacent plants were not in contact until the month of July, by which time the plants were almost fully developed and in a state of active tuber formation.

Presumably then the direction of major food transport was towards the tubers at the time infection took place. If it be true that the main movement of virus is with the food stream, it is not difficult to see why under the circumstances the virus should be carried from the site of inoculation on the leaf to the tuber without necessarily spreading to other parts of the plant.

The failure to become systemic in the foliage may also account for the frequency with which the tubers from the field-infected plants produced healthy as well as diseased shoots. Such a phenomenon has not been observed in the case of tubers from plants with diseased foliage. It is generally believed that the mosaic viruses multiply chiefly in the leaves, and it has been shown that downward movement of virus in a potato stem is not dependent upon multiplication. It may be suggested, therefore, that in the plants under discussion there was probably slight multiplication of virus at the site of inoculation until the vascular tissue was reached, and that the quantity of virus thus formed was carried to the tubers in the food stream without further reproduction. The amount of virus reaching the tubers of late-infected plants might therefore be extremely small. Furthermore, the results of inoculation and serological tests with expressed juice of tubers from plants systemically infected with X show that the quantity of virus in tuber juice is much less than that in leaf sap; which suggests that there may be little or no multiplication of virus X in the tubers. If these suppositions be correct, it is only to be expected that the quantity of virus in the tubers of late-infected plants would be insufficient to cause infection of every developing sprout. Only when an infected sprout formed green leaves might the virus multiply freely, and from such a source other shoots on the same tuber would eventually become infected, provided there was continuity of sap flow between them.

References in the Literature to Strains of X similar to the Severe Strain under investigation.

The X^N strain which Salaman (29) described in 1938, and which occurred originally in plants of the variety Majestic grown in Scotland, corresponds closely to the writer's severe strain in its symptoms on several varieties of potato and on other solanaceous hosts, as well as in its physical properties. Furthermore, referring to X^N in potato Salaman observed that "when a stock is carried on by tuber in the glasshouse it is not rare to find that in subsequent years in one or other plant of a clone a more or less complete clinical recovery has taken place. In such a case only X^G may be found." This passing reference indicates that the phenomenon of recovery is associated with X^N as well as with the strain described in the present paper.

There are certain points, however, upon which the writer's severe X differs from X^N as described by Salaman (29). For example, X^N was reported to be

less readily communicable by grafting than by sap inoculation, infection sometimes failing even in cases where union between scion and stock was good. Such has not been the experience in grafting experiments with severe X. It was also stated that X^N-infected tubers of Majestic and other varieties might in the second season give rise to healthy plants, fully infected ones, or fail to sprout. In the case of severe X, tubers from systemically infected plants invariably gave rise to diseased plants, and sprouting was not affected. Furthermore, leaf drop is predominantly a 1st year symptom of the writer's strain, while Salaman describes it as a conspicuous 2nd year symptom in the case of X^N.

In certain varieties of potato the symptoms produced by X^N differed from those of severe X. For example, Arran Banner, Ballydoon, and Doon Star, were stated to react with a relatively mild mottle; but the results were based on infection of only one experimental plant in each case. The reaction of Eclipse to X^N was found to be variable, this variety responding either with a mild interveinal mottling or else behaving as a carrier. It was believed, also, that mutation of X^N to X^S occurred during passage through Eclipse. These experiments, however, were confused by the fact that part, at least, of the clone of Eclipse used was already infected with a latent strain of X. Virus-free Eclipse (12 plants) infected with severe X in the present work reacted in the same manner as other tolerant varieties. With regard to intolerant varieties, Salaman found that the reaction of King Edward to X^N differed from that of Arran Crest and Epicure. No symptoms followed sap inoculation, but 2 plants behaved as carriers. Only a small percentage of plants was infected by grafting, but these reacted with top-necrosis. It was also suggested that X^N was converted into a strain of lower virulence by passage through this variety. In the writer's experiments King Edward, Arran Crest, and Epicure all reacted similarly to severe X.

The differences outlined above, if established, would be sufficient to distinguish the two strains of X. It seems likely, however, that some of them, at least, would be eliminated if the two strains were compared simultaneously under identical conditions.

Bald and Pugsley (1) reported that a necrotic strain of X, which they equated with Salaman's X^N, is sometimes a component of mixtures of X carried in Australian commercial potato stocks. It is also possible that similar strains occur in American varieties of potato, judging by the symptoms produced by certain of the American severe ringspot and "virulent latent" strains of X in tobacco, tomato, and other solanaceous hosts. The same may be said of strains isolated by Köhler ((21) (23)) from German potato stocks. Exact comparison is difficult without knowing the reactions of these various strains on appropriate virus-free varieties of potato.

The potato ringspot virus described by Putnam (28), which Salaman equates with X^N, also approaches the writer's severe X strain in its symptoms

on various non-potato solanaceous hosts as well as in its physical properties. In the variety President, however, Putnam's virus failed to produce symptoms; and unless the plants used were, like Green Mountain and Bliss Triumph, already infected with latent X, this reaction would constitute a clear distinction between his virus and both the X^N and severe X strains.

The D strain of virus X described by Bawden (3) resembles the writer's severe strain in its symptoms on a certain number of potato varieties. It causes top-necrosis, however, in Arran Consul and Majestic, is carried by Arran Banner, Champion, and other varieties, and also differs from the writer's strain in its symptoms on *Datura stramonium*, tomato, and *Nicotiana glutinosa*.

Discussion.

The most interesting feature of the severe X disease described in the present paper is the consistent manner in which its symptoms become attenuated in affected potato stocks during the course of a few years. This recovery is distinct from the recovery of *Nicotiana* spp. from Tobacco Ringspot recorded by Price (27), in which the plants become carriers of the virus.

Two suggestions may be put forward to explain the reduction in virulence of the severe X disease: (1) That the original inoculum contains a mixture of severe and mild strains and that the mild strain multiplies with greater rapidity than the severe one and gradually displaces it from the stock; and (2) that the severe strain mutates to the mild form within the tissues of the potato plant. The chief difficulty in accepting the former explanation is that there is no evidence that the severe strain multiplies or moves through its hosts less rapidly than the milder strains. On the contrary, it is highly infectious and becomes systemic in potato and other hosts with a rapidity at least equal to, if not greater than, that of mild X. Bald and White (2) consider that the ability to multiply more rapidly than milder strains is an attribute of severe strains of potato virus X, although they visualise the possibility that a necrotic strain may limit itself by killing the sites for its own multiplication. In the disease under discussion, however, the most severe necrosis of the foliage occurs in the first year of infection, whereas recovery does not take place before the end of the second or third year. This indicates that the damage done to the tissues is not accountable for the limitation of this particular strain in potato. There are other reasons, too, for doubting that the attenuation is due simply to the settling out of a mild strain from an original mixture of mild and severe X. In Orinoco tobacco the severe strain tends to be strictly localized, whereas milder strains become systemic and produce mosaic symptoms. If the inoculum contained a mixture of mild and severe strains, a segregation thereof might reasonably be expected in this host, even allowing that a certain amount of destruction of a mild strain might occur in the lesions produced by the

severe form. No indication of the presence of a mild strain was obtained in this way. Furthermore, it has already been mentioned that when the severe strain was introduced by graft to potato plants infected with mild X, the plants to which the tubers gave rise in the following year differed in appearance from control plants infected with the severe strain only.

These considerations have led to the tentative conclusion that the recovery of potato stocks from the severe disease is not due simply to the sorting out of an original mixture of viruses, but results from the mutation of the severe strain to a mild form. Salaman (29) hesitated to invoke mutation in explanation of the recovery which he observed in X^N-infected potato plants in the glasshouse, suggesting that the change was due to the over-running of the plants by a mild strain of X present in admixture with the severe strain from the start. This view, however, is not in keeping with his experimental evidence, which indicated that X^N occurred originally in potatoes in the pure state. Apart from this, it is clearly difficult to explain the presence of mixtures of X strains in field plants except on the basis of mutation. So far as is known, the various strains of X are mutually protective, for Köhler's (22) statement that the mottle strains do not protect against the ringspot forms has been contradicted by Salaman (29) and also conflicts with the results obtained in this laboratory. The only known method of spread of virus X is by contact between diseased and healthy plants which is equivalent to sap inoculation under artificial conditions. Theoretically, then, a plant already infected with one strain of X should not become infected with a second strain under field conditions. Salaman (29) suggested that field plants infected with mixed strains might have received such strains either simultaneously, in a single inoculation, or in two or more infections at short intervals of each other; but this theory obviously implies the eixstence of a vector of virus X, of which there is at present no evidence.

That plant viruses do mutate is now generally accepted, but in the majority of cases recorded the altered strain has been localized, usually in small spots on the leaves. Wholesale changes are unusual and are certainly not a feature of mild X diseases such as simple mosaic and mosaic *ex* C (10), both of which have been cultivated at Glasnevin in many successive generations of potato without displaying any permanent general change in symptoms. The severe strain of X thus differs markedly from the common mild strains in its instability in potato. This consideration leads one to speculate regarding the origin of the severe strain. As there is no evidence of spontaneous generation of a virus the simplest theory would be that it arises by mutation within the potato plant from a milder strain and multiplies to the virtual exclusion of the latter. This view, however, is not supported by evidence obtained in the present investigation, for as yet no case of reversion from the mild to the severe form has been observed. Furthermore, Salaman (29) records that all strain reversions observed by him were from the more to the less virulent form and

efforts to increase the virulence of mild strains artificially were unsuccessful. There is also the fact, which is possibly significant, that the severe strain cropped up on several occasions in seed potato stocks raised originally from virus-free tubers and which might reasonably be expected to be more or less free from X (12). In the circumstances, one might go so far as to suggest that the severe strain is not native, as such, to the potato, but arises by passage of a milder strain through another host resulting in intensification; and that it tends naturally to revert to the mild form within the potato environment. It is true that there is no evidence that passage through solanaceous weeds alters the character of the severe strain; besides, such weeds are uncommon in Irish potato fields. The fact, however, that *Lamium hybridum* can be systemically infected suggests that there may be other non-solanaceous hosts of X which are as yet undiscovered and which might have an intensifying effect on mild strains of this virus.

Salaman (29) was the first to attempt a survey of the various strains of X occurring in potatoes in England. The strains are defined by their reactions on a variety of solanaceous hosts, principally the tobacco variety White Burley and *Datura stramonium*. Three of the six strains which Salaman describes, viz. X^H, X^N, and X^D, were found in the pure state in potato; the remaining three, X^G, X^L, and X^S, were isolated by subjecting the virus from infected potatoes to selective inoculation through several generations of tobacco, in the belief that the plants contained mixtures of strains. The drawback of this method of analysis is that it neglects the possibility of mutation within the tissues of the tobacco. If the development of new strains in isolated spots on tobacco leaves were to occur, in the case of virus X, with the same frequency as in the case of the tobacco mosaic virus, there would be no certainty that the strains isolated by this technique occurred as such in the original potato. Nevertheless, no better method is at present available, and Salaman states that one, at least, of the strains isolated in this manner (X^S) was also isolated direct from potato; while another of them (X^G) corresponds closely with the simple mosaic (10) common in potatoes in this country.

The grouping of X strains into mottle and ringspot forms according to symptoms on tobacco, originally adopted in America, is not sufficiently discriminating. It may even be unsound, for a strain of X from this laboratory, diagnosed as "mottle" by Koch and Johnson (20), is capable of inducing mild ring formation in tobacco under suitable environmental conditions, which suggests that there is no hard and fast line between mottle and ringspot symptoms in this host. Regarding the other basis of distinction between the two groups, viz. thermal death point, it is doubtful whether the end points obtained in experiments with crude saps are sufficiently sharp to render a difference of 2° C. a reliable diagnostic feature. It is not even certain that such a difference exists, as Salaman (29) records a thermal death point of 68° C. for the G and L strains of X which he equates with Koch's (19)

"mottle"; while the same figure is given by Koch (19) as the thermal death point of "ringspot."

Chester (7) has demonstrated the existence of antigenic differences between three different strains of X designated potato mottle, potato ringspot, and masked potato mottle. It is possible, therefore, that serological methods may furnish the most exact means of distinguishing the various strains of virus X following further experimentation along these lines.

In the meantime it is clear that identification of X strains in general cannot be made solely on the basis of symptoms in tobacco or *Datura stramonium*. Such strains, for example, as X^G (29), X^D (3), and the Up-to-Date top-necrosis virus (9) are not distinguishable with any degree of certainty on these hosts. It is desirable, therefore, in attempting to identify strains of X, to use appropriate varieties of virus-free potato in conjunction with other solanaceous hosts.

The strains of X at present known to occur naturally in potato, and their characteristic reactions may be summarised as follows:—

X^B (**Up-to-Date top-necrosis virus** ((9) (14)).—Top-necrosis in President potato. Mottle in Arran Crest and Epicure. Mild mosaic in *Datura stramonium* and tobacco.

X^D ((3) (29)).—Severe foliar necrosis in President and certain other potato varieties. Top-necrosis in Arran Crest and Epicure. Mild mosaic in *Datura stramonium* and tobacco.

X^H (29).—Carried symptomlessly in President. Top-necrosis in Arran Crest and Epicure. Carried symptomlessly in *Datura stramonium* and tobacco. Mosaic and necrosis in *Capsicum annuum*.

Chester's (7) masked potato mottle is probably the same strain.

X^G (29) **or Simple Mosaic** (10).—Carried symptomlessly or very mild mottle in President potato. Top-necrosis in Arran Crest and Epicure. Mild mosaic in *Datura stramonium* and tobacco.

A slightly stronger form of X^G occurs, typified by the mosaic *ex* C described from this laboratory (10) and distinguishable only by the somewhat greater intensity of the symptoms in the non-potato hosts.

X^S (29).—Interveinal mosaic and slight foliar necrosis in President potato. Top-necrosis in Arran Crest and Epicure. Severe local and systemic necrosis and mosaic in *Datura stramonium* and tobacco.

X^N (29).—Severe foliar necrosis in President and other potato varieties, including a number which do not react similarly to X^D. Top-necrosis in Arran Crest and Epicure. Severe local and systemic necrosis and mosaic in *Datura stramonium* and tobacco.

The severe strain described in the present paper may be regarded as similar to X^N with the qualifications mentioned in the text.

Putnam's Yellow Mottle Virus (28).—Yellowish interveinal mottling on President potato. Indefinite mottling on tobacco. Characteristic yellow mosaic

in tomato. Thermal death point 73° C. The identity of Putnam's virus as a strain of X has not yet been generally accepted (29).

The above list is a slightly amended form of that drawn up by Salaman (29). Excepting the Yellow Mottle virus, all the strains included are related immunologically and serologically and have a thermal death point of 68°–70° C.

The fact that virus X appears not to be transmitted except by contact between diseased and healthy plants implies that infection with this virus, even in "field susceptible" varieties, should be easy to avoid in seed potato stocks propagated from X-free tubers. According to present knowledge one might reasonably assume that contamination of such stocks in the field could only arise from infected "volunteer" plants within the crop; or from perennial weeds if any such were hosts of virus X. Control of X in partially infected crops by rogueing of diseased plants should be effective, provided "contact" plants are also removed, whether or not these show disease symptoms or react positively to other tests for the presence of X. This precaution is important in view of the fact that X can reach the tubers of a potato plant without necessarily infecting the foliage to any appreciable extent. The best method of avoiding X naturally lies in the use of "field immune" varieties as advocated by Cockerham (13); but the number of such varieties which are desirable in other respects is as yet extremely small.

Summary.

A disease occurring naturally in seed potato stocks and characterised by symptoms of severe mosaic and necrosis was found to be due to a strain of virus X.

The symptoms of the severe strain in 32 varieties of potato are described. In all except three, which reacted with top-necrosis, an initial phase of foliar necrosis and leaf drop was followed by severe mosaic and stunting in the current year of infection.

The disease in potato was characterised by a striking decrease in virulence, which took place usually in the 3rd year of infection of the clone, and occurred consistently in all varieties. The recovered plants contained a mild strain of X. Reasons are advanced for the theory that the recovery is due to mutation of the severe strain to a mild form.

In other solanaceous hosts symptoms consisted of local and systemic necrosis, severe mosaic, and stunting.

Of a large number of non-solanaceous plants inoculated with the severe strain, only one (*Lamium hybridum*) became systemically infected. *Veronica agrestis*, beetroot, and mangold, reacted with local lesions.

The physical properties and serological reactions of the severe strain are similar to those of other X strains. Plants infected with a mild strain are immune to infection with the severe strain by sap inoculation, but not by grafting.

The U.S.D.A. Seedling 41956 is immune to infection with the severe strain. In double-grafted plants over 2½ months old, severe X passed freely downwards through intermediate scions of 41956. Upward movement in plants of the same age was inhibited by the presence of the immune variety. The significance of these results in regard to movement of virus X within the potato plant is discussed.

In field experiments considerable spread of the severe strain resulted from contact between diseased and healthy plants of a tolerant variety. That no insect vector was involved was shown by the absence of infection in plants grown in proximity to, but not actually touching, disease sources.

Disease symptoms were rarely observed in the current year of infection in the field. The production on the same tuber of healthy and diseased shoots following field infection is discussed.

Plants of intolerant varieties of potato failed to become infected by contact with disease sources in the field.

The relation of the severe strain to Salaman's X^N is examined; and the problem of the identification of X strains is discussed.

EXPLANATION OF PLATES.

Plate 15.

Fig. 1.—Potato *var.* Majestic three weeks after inoculation with the severe strain of X.

Fig. 2.—Healthy plant *var.* President and plant infected by core-graft with severe X. The withered lower leaves of the diseased plant are obscured in the photograph.

Fig. 3.—Top of President plant from clone in 3rd year of infection, showing recovery in apical growth.

Plate 16.

Fig. 4.—Potato plant *var.* Kerr's Pink in 2nd year of infection wtih severe X, showing recovery in one shoot.

Fig. 5.—Leaf of tomato infected with severe X, showing characteristic interveinal necroses.

Fig. 6.—Field plants of President potato. Left, 4th year of infection; and right, 2nd year of infection of clone.

Plate 17.

Fig. 7.—*Datura stramonium* plants of same age, 13 days after inoculation from potato plants (*a*) in 3rd year of infection showing recovery (left), and (*b*) in 1st year of infection with the severe strain.

Fig. 8.—*Nicotiana glutinosa* 13 days after inoculation with the severe strain.

Fig. 9.—Tobacco plant (*var.* Orinoco) infected with the severe strain. The top of this plant was virus-free.

FIG. 1.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

Photographs by G. H. MCLEAN.

FIG. 7.

FIG. 8.

Photographs by G. H. McLEA

FIG. 9.

REFERENCES.

(1) BALD, J. G., and PUGSLEY, A. T.—Coun. Sci. Ind. Res. (Aust.), Pamph. 110, 1941.
(2) ——— and WHITE, N. H.—J. Coun. Sci. Ind. Res. (Aust.), **15**: 300–306, 1942.
(3) BAWDEN, F. C.—Proc. Roy. Soc., Lond., B, **116**: 375–395, 1934.
(4) BENNETT, C. W.—Bot. Rev., **6**: 427–473, 1940.
(5) BURNETT, G., and JONES, L. K.—Wash. Agr. Exp. Sta. Bull., 259, 1931.
(6) CHESTER, K. S.—Phytopath, **25**: 686–701, 1935.
(7) ——— *Ibid.*, **26**: 778–785, 1936.
(8) ——— *Ibid.*, **27**: 722–727, 1937.
(9) CLINCH, P. E. M.—Sci. Proc. Roy. Dublin Soc., **23** (N.S.): 18–24, 1942.
(10) ——— and LOUGHNANE, J. B.—*Ibid.*, **20** (N.S.): 567–596, 1933.
(11) ——— and MURPHY, P. A.—*Ibid.*, **21** (N.S.), 431–448, 1936.
(12) ——— *Ibid.*, **22** (N.S.), 17–31, 1938.
(13) COCKERHAM, G.—Scot. Journ. Agric., **22**: 1–11, 1939.
(14) ——— Anns. App. Biol., **30**: 338–344, 1943.
(15) GRATIA, A., and MANIL, P.—C. R. Soc. Biol., **117**: 490–492, 1934.
(16) HANSEN, H. P.—Tidsskr. Planteavl., **42**: 641–681, 1937.
(17) JOHNSON, J.—Wis. Agr. Exp. Sta., Bull. 63, 1925.
(18) JONES, L. K., ANDERSON, E. J., and BURNETT, G.—Phytopath. Zeitschr., **7**: 93–115, 1934.
(19) KOCH, K.—Phytopath., **23**: 319–342, 1933.
(20) ——— and JOHNSON, J.—Anns. App. Biol., **22**: 37–54, 1935.
(21) KÖHLER, E.—Phytopath. Zeitschr., **7**: 1–30, 1934.
(22) ——— *Ibid.*, **10**: 31–41, 1937.
(23) ——— Naturwissenschaften, **49**: 828–830, 1935.
(24) LOUGHNANE, J. B., and MURPHY, P. A.—Sci. Proc. Roy. Dublin Soc., **22** (N.S.): 1–15, 1938.
(25) MURPHY, P. A., and MCKAY, R.—*Ibid.*, **20** (N.S.): 227–247, 1932.
(26) ——— *Ibid.*, **20** (N.S.): 347–358, 1932.
(27) PRICE, W. C.—Contrib. Boyce Thompson Inst., **4**: 359–403, 1932.
(28) PUTNAM, D. F.—Canad. J. Res., C, **15**: 87–107, 1937.
(29) SALAMAN, R. N.—Phil. Trans. Roy. Soc., Lond., B, **229**: 137–217, 1938.
(30) SCHULTZ, E. S., CLARK, C. F., RALEIGH, W. P., STEVENSON, E. J., BONDE, R., and BEAUMONT, T. H.—Phytopath., **27**: 190–197, 1937.
(31) SPOONER, E. T. C., and BAWDEN, F. C.—Brit. J. Exp. Path., **16**: 218–230, 1935.
(32) WILLIS, J. C.—A Dictionary of the Flowering Plants and Ferns, Cambridge Univ. Press, 1919 (4th Ed.).

No. 29

"STUDIES IN PEAT."

Part 13.

MONA WAX AND ITS CONSTITUENTS AS EMULSIFYING AGENTS.

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In Part 12 of this series (1) it was shown that Mona wax, as extracted from Irish peat by such solvents as petrol-alcohol, trichlorethylene, etc., is capable of promoting the emulsification of water in certain hydrocarbon liquids. The water-in-oil (W/O) emulsions sponsored by the wax can contain a high percentage of water, and even when the concentration of the water has a value of 75 per cent. the emulsions are characterised by a good degree of permanence. The investigations described in the present paper will show *inter alia* that the various constituents of Mona wax have not equal values as emulsifying agents, and that the nature of the oil exercises an influence on the character of the emulsion formed when the wax is used as the agent. The effect of heat on wax-sponsored emulsions and the mechanism of the changes caused by increased temperature will also be described. In addition, the effects produced due to the addition of certain electrolytes to the water to be emulsified will be discussed.

Experimental.

Emulsions were in all cases prepared by the intermittent addition intermittent shaking method (1). Such a procedure permitted the comparison of the characteristics of different emulsions formed under approximately similar conditions. Tests for type were carried out as indicated (*loc. cit.*). Behaviour on ageing was judged by allowing the emulsions to stand in corked vessels at room temperature with, in most cases, gentle mixing at irregular intervals to redistribute creamed material. The contents of vessels were examined occasionally both by unaided vision and (after suitable dilution of representative samples with the oil) under the microscope, no cover-glass being used (1). The results of the investigations were as follows:—

Effectiveness of Mona wax as emulsifying agent according to the mode of its extraction from peat and its subsequent treatment (2). The following waxes were examined:—(A) a wax extracted from peat by trichlorethylene (97 per cent. soluble in "white spirit"), (B) a wax extracted from peat by trichlor-

ethylene, and rendered 100 per cent. soluble in "white spirit" by chemical treatment (2), (C) a wax extracted from peat by a mixed solvent (60 per cent. petroleum spirit and 40 per cent. isopropyl alcohol), and subsequently treated with 5 per cent. chlorosulphonic acid. In each case 0·2 g. of the wax was warmed with 20 ml. of liquid paraffin. In the case of wax (A) traces of the wax remained undissolved. In cases (B) and (C) the waxes dissolved completely. 60 ml. of distilled water was then emulsified in the resultant liquid, the temperature in each case being maintained at approximately 40° C. throughout the process. The following observations were made:—

Wax (A): Apart from the fact that the traces of insoluble wax-matter settled on the walls of the container, this wax gave the most viscous and qualitatively the best emulsion. Very little deterioration of the system occurred on three months' standing.

Wax (B): This wax gave an emulsion of good permanence and of almost paste-like consistency. On account of the absence of insoluble matter this wax was used in many of the investigations reported in this paper.

Wax (C): This wax promoted a good emulsion which, on standing for a period of three months, underwent only slight deterioration.

Characteristics of the emulsions formed in selected oils, using Mona wax (extracted from peat by trichlorethylene, and rendered 100 *per cent. soluble in hydrocarbon) as agent.* The oils chosen were liquid paraffin, benzene, toluene, xylene, and sunflower oil. In each case 0·1 g. of the wax was dissolved in 20 ml. of the warmed oil, and the distilled water added as already indicated, the temperature being kept at approximately 40° C. during the addition. The observations made were as follows:—

Liquid paraffin. A very viscous emulsion resulted from the use of 60 ml. of distilled water. The permanence of a water-in-liquid-paraffin system with the wax as emulsifying agent is being separately investigated by the technique of size-frequency analysis.

Benzene. A viscous emulsion was formed when 60 ml. of distilled water was added. The emulsion could initially be submitted to continuous rapid shaking without showing signs of undue deterioration. On ageing, however, deterioration of the system became evident. On allowing the emulsion to stand undisturbed for a period of two months, globules of water, large enough to be visible to the unaided eye, were observed in the system, and a bulk quantity of water had collected at the base of the containing vessel.

Toluene. The emulsification of 60 ml. of water in toluene resulted in a product which was qualitatively better than that obtained when benzene was used. Like the water-in-benzene emulsion, the emulsion in toluene could be submitted. when freshly made, to rapid continuous shaking without evident deterioration. On ageing the system behaved in a manner analogous to that observed in the water-in-benzene system. An emulsion formed under similar circumstances of 75 ml. of water in 20 ml. of toluene (which originally contained 0·1 g. of the wax) showed similar characteristics of stability and permanence,

but even after four months of standing no bulk quantity of water had collected at the bottom of the vessel. 0·4 g. of the wax was dissolved in 20 ml. of toluene, and an investigation made of the possibility of forming a very concentrated emulsion of water in toluene from the resulting solution, the mode of preparation and the temperature being as aforementioned. When 90 ml. of water had been added the emulsion broke.

Xylene. This, as a dispersion medium, was not nearly as effective as toluene or benzene. The emulsion given by 60 ml. of water was, by visual examination, of poor quality though fairly viscous. Continuous rapid shaking of the emulsion caused separation of some of the water in large drops. On ageing the system deteriorated rapidly.

Sunflower oil. This oil was chosen as a typical non-hydrocarbon liquid. The emulsion, which contained 60 ml. of dispersed water, contained fine globules when freshly prepared. It was of paste-like consistency. This emulsion was allowed to stand undisturbed for observation of its behaviour on ageing. It deteriorated more rapidly than the water-in-benzene and water-in-toluene emulsions, but less rapidly than the water-in-xylene emulsion.

Effect of heat on an emulsion of water in liquid paraffin. An emulsion was prepared containing 60 ml. of dispersed distilled water, 20 ml. of liquid paraffin, and 0·2 g. of wax (extracted from peat by trichlorethylene, and rendered 100 per cent. soluble in hydrocarbon; m. pt. of wax 75° C.) as emulsifying agent. The emulsion was allowed to stand overnight, and then divided into three parts. The separate portions were placed in test-tubes, and the vessels closed with corks. One of the containers was placed in a water-bath maintained at 90° C., another in a water-bath maintained at 80° C., and the third in a bath kept at 65° C. After 15 minutes it was observed that the phenomena of creaming and breaking were occurring simultaneously in the two systems kept at the higher temperatures, that of breaking occurring more rapidly in the system at 90° C. than in the system maintained at 80° C. After thirty minutes the volume of water which had collected as a bulk layer at the bottom of the test-tube was about ·5 ml. from an emulsion of total volume 25 ml. in the case of the sample heated at 90° C., and somewhat less in the case of the sample at 80° C. The tubes and contents were then submitted to six single shakes, and the emulsion in each case reformed temporarily, only to break again rapidly to the same extent as before on resting in the appropriate water-bath. Again it was noted that the emulsion at 90° C. deteriorated more rapidly than that at 80° C. The emulsion in the 80° C. water-bath was then removed and cooled to 70° C., shaken six times, and maintained at the new temperature. The shaking again caused the emulsion to reform temporarily, but again water separated as a bulk layer on resting. In the case of the emulsion at 65° C. some breaking had occurred after one hour, but to a much less extent than at the higher temperatures.

An emulsion of 16 ml. of distilled water in 24 ml. of liquid paraffin, with 0·2 g. of the wax (of the same nature as that just mentioned) was prepared at

room temperature (16° C.), and its behaviour on ageing was observed at intervals by examination of the gently-mixed emulsion, suitably diluted, under the microscope. It was some weeks before the interfacial films became distinct.

Mechanism of the breaking due to heating. The permanence of water-in-liquid-paraffin emulsions sponsored by the wax is due to the envelopment of the water-globules by interfacial wax-films (1). When the wax itself dissolves in liquid paraffin it gives a yellow-brown colloidal solution. When a water-in-liquid-paraffin emulsion creams, the clear supernatant layer of liquid consists normally of a very dilute solution of the wax in liquid paraffin, as judged from the very faint yellow colour of the layer in question. When, however, the creaming was accompanied by breaking due to elevation of the temperature, the supernatant liquid was brown-yellow in colour, indicating that the breaking was associated with re-peptization (3) of the film-material by the liquid paraffin.

Effect of the presence of electrolytes in the water on the character of the emulsion formed. The following substances were dissolved in distilled water so as to give solutions of the concentrations indicated:—Sodium chloride, 0·1 per cent.; calcium chloride, 0·1 per cent.; magnesium chloride, 0·1 per cent.; magnesium sulphate 0·1 per cent.; aluminium sulphate, 0·1 per cent.; sodium oleate, 1 per cent.; hydrochloric acid, N/10; sodium hydroxide, N/10. Attempts were made to prepare, in each case, an emulsion containing 20 ml. of liquid paraffin, 0·2 g. of the wax (100 per cent. soluble in hydrocarbon), and 60 ml. of the solution of electrolyte, the temperature of preparation being 40° C. Results:—

With sodium chloride, calcium chloride, magnesium chloride, and magnesium sulphate solutions the products were more viscous than that obtained when distilled water was used, but the final emulsions were inferior to that produced when water alone was the internal phase. When these emulsions were allowed to rest undisturbed, deterioration set in. After two months of ageing, bulk-quantities of water or aqueous solution had collected at several points throughout the systems, but the unbroken parts of the emulsions, which were appreciable, still preserved a highly pasty consistency.

Aluminium sulphate. The final product was again highly viscous and somewhat resembled that produced when a solution of sodium chloride was employed. During the later stages of the preparation of the emulsion (after 35 ml. of the electrolyte solution had already been emulsified), difficulty was experienced in bringing about emulsification of the solution, unemulsified liquid being visible in the system after the shakings. When the system had been standing for a period of two months, water or aqueous solution had collected at several points throughout the mass, but the pasty consistency of the remainder of the system was maintained.

Sodium oleate. The final product was a "multiple" system in which water or an aqueous solution was the primary external phase. The product was of low viscosity and creamed (upwards) rapidly.

Hydrochloric acid solution. After the emulsification of 50 ml. of the

aqueous solution, additional quantities of the dilute acid could not be emulsified by the method of preparation followed. The emulsified portion, in contact with the excess unemulsified liquid, remained reasonably permanent for four weeks.

Sodium hydroxide solution. When 50 ml. of the aqueous solution had been added the system, although viscous, was not of the same highly pasty consistency as that obtained, for example, when 0·1 per cent. sodium chloride solution was used. The remaining 10 ml. (50 ml. to 60 ml.) could not be emulsified well. Apart from the separation of this excess volume of liquid, the system underwent no evident gross change on standing undisturbed for a period of three weeks.

Individual constituents of the wax as emulsifying agents. The following constituents of the wax were tested for effectiveness as emulsifying agents: (A) the resinous matter which is soluble in cold alcohol (4 and 5), (B) the constituent removed from the wax (extracted from peat by trichlorethylene and rendered 100 per cent. soluble in hydrocarbon) by means of boiling alcohol (95 per cent.) which is precipitated from the alcoholic solution on cooling, (C) the matter in this wax which is not soluble in boiling alcohol, (D) the "total acids" in the wax (6). The following observations were made:—

(A) (resinous matter). 0·1 g. of the resin was dissolved in 20 ml. of liquid paraffin, and distilled water was added with shaking in the same manner as before, the temperature being maintained at 40° C. It was found impossible to emulsify more than 30 ml. of the water. The experiment was repeated using 0·2 g. and 0·25 g. of the resin in the same volume of liquid paraffin, the conditions of preparation being the same as before. With 0·2 g. of the resin about 30 ml. of water was emulsified, and with 0·25 g. of resin it was possible to emulsify 40 ml. of water. These emulsions were allowed to stand undisturbed in contact with the excess added water, for a period of three months. Rapid deterioration set in.

(B) (material dissolved by hot ethyl alcohol and precipitated on cooling the solution). 0·1 g. of this material, in 20 ml. of liquid paraffin, allowed 40 ml. of water to be emulsified, the temperature being maintained at 40° C. during the preparation. On allowing the system, with an excess of added water, to stand undisturbed for three months, it was found that about 15 ml. of the water originally emulsified still remained in the emulsified condition.

(C) (material insoluble in hot alcohol). This material was obtained by extracting the wax to exhaustion with 95 per cent. ethyl alcohol. The wax had to be refluxed with the alcohol a large number of times to remove completely the alcohol-soluble matter. 27·5 per cent. of the wax remained as insoluble pitch-like matter. This material was not peptized very readily by hot liquid paraffin. 0·1 g. of this substance, in 20 ml. of liquid paraffin, readily allowed the emulsification of 60 ml. of distilled water, the temperature of preparation being 40° C. The emulsion formed was of good quality and of paste-like consistency. An emulsion formed under similar circumstances of 60 ml. of

water in 20 ml. of liquid paraffin, with 0·2 g. of the alcohol-insoluble material as emulsifying agent, was so viscous when cold that it could not be poured from the container. The emulsions promoted by this constituent are being further investigated.

(D) (total acids). 0·1 g. of the total acids in 20 ml. of liquid paraffin gave a thick coarse emulsion when as much as 35 ml. of water had been added, the temperature of the preparation being 40° C. More than 35 ml. could not be emulsified under the conditions chosen. When the system (including the excess water) was allowed to stand undisturbed for a period of three months, only 10 ml. of the water originally emulsified had not separated in the bulk layer at the bottom of the container during this period of ageing.

Investigation of sodium salts of "total acids" and of resin acids as possible agents for the production of oil-in-water emulsions. With the exception of the "multiple" type of emulsion formed when the aqueous phase contained sodium oleate, all of the emulsions thus far described were of the water-in-oil type. Sodium and potassium soaps usually sponsor the oil-in-water type of emulsion. It was decided to determine whether the sodium salts of the total acids and of the resin acids had any value as emulsifying agents for the production of O/W emulsions.

A weighed quantity of the total acids (5) was dissolved in 95 per cent. ethyl alcohol, and an alcoholic solution of an estimated quantity of sodium hydroxide was added. The mixture was refluxed for 1 hour, and then allowed to cool. The soap which separated was filtered, washed with a little cold alcohol, then washed with ether, and finally dried. The soap was then dissolved in distilled water so as to give a solution which was estimated to have a concentration of approximately 0·01N. Of this solution 12 ml. was taken. For comparison purposes, 12 ml. of 0·01N aqueous sodium oleate was taken in another vessel. In either case 8 ml. of kerosene was added in 0·5 ml. quantities up to 3 ml., and then in 1 ml. quantities up to the 8 ml., with intermittent shaking at room temperature. The sodium salts of the acids appeared to have no value as emulsifying agents.

The product obtained when the resin (4 and 5) was (as far as possible) dissolved with the minimum amount of sodium hydroxide solution was compared under similar circumstances with the sodium compound of colophony. The colophony complex was a better emulsifying agent.

Discussion of Results.

The fact that the nature of the oil exercises an influence on the character of the emulsion formed is not surprising. Numerous workers have commented on the phenomenon of specificity in emulsification processes (6). The effect of heat on the concentrated emulsion chosen suggests that, while emulsification is often facilitated by slight elevations of the temperature, the optimum temperature for the preparation of W/O emulsions promoted by the wax would

lie below 60° C. The effects observed when the aqueous phase contained electrolytes might have been due to physico-chemical influences of the electrolyte or, in certain cases, to the methood of emulsion-preparation which was followed. Mere shaking of a system is, at best, an inferior method for the preparation of emulsions. In the cases where the aqueous phase contained dissolved inorganic salts, the resultant systems were already highly viscous before all of the aqueous solution had been added. The high viscosity already reached may have been a factor in determining the character of the final system. When sodium oleate was present in the aqueous phase, the "multiple" system obtained was due to the antagonistic influence of this soap (which, under the given conditions, favours the O/W type of emulsion). The superiority of the hot-alcohol-insoluble matter as an emulsifying agent is noteworthy.

Summary.

Mona wax (both untreated and treated so as to be 100 per cent. soluble in hydrocarbon) has been shown to be effective in sponsoring certain W/O emulsions.

The nature of the oil is a factor in determining the character of the emulsion formed. When the oil was toluene it was found possible, following the mode of preparation used, to prepare an emulsion containing more than 81 per cent. of dispersed water, using 0·4 g. of wax, per 20 ml. of toluene, as emulsifying agent.

The effect of heat on an emulsion of water in liquid paraffin with the wax as emulsifying agent has been studied, and it has been observed that at temperatures above 60° C. the breaking which occurs is associated with re-peptization of the film material by the liquid paraffin. It is considered that the optimum temperature for the preparation of such emulsions lies below 60° C.

The effect of the presence, in the water, of certain electrolytes has been examined. A solution of sodium oleate gave rise to a "multiple" system.

The components of the wax have different values as emulsifying agents. The constituent which gave the best result was the fraction insoluble in boiling 95 per cent. ethyl alcohol.

REFERENCES.

(1) Aherne and Reilly.—Sc. Proc. Roy. Dubl. Soc., 1944, **23**, 247.
(2) Reilly and Kelly.—Industrial Research Council (Éire) Bulletin No. 3, 1943.
(3) The term "peptization" is here used in the wider sense favoured by Bancroft, J. Phys. Chem., 1916, **20**, 85.
(4) Reilly and Emlyn.—Sc. Proc. Roy. Dubl. Soc., 1940, **22**, 267.
(5) Reilly and Wilson.—*Ibid.*, 1940, **22**, 321.
(6) See, for example, Kernot and Knaggs, J. Soc. Chem. Ind., 1928, **47**, 96 (Trans.).

No. 30.

A RAPID FERMENTATION METHOD FOR THE PRODUCTION OF CALCIUM CITRATE AND CALCIUM GLUCONATE FROM BEET MOLASSES.

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Introduction.

Since Wehmer's original patents in 1894 a great deal of research on the citric-acid-producing fungi has been carried out, resulting in the establishment in several countries of fermentation industries for the production of citric acid. The history of this fermentation is well reviewed by Wells and Herrick (1938). The details of the processes at present in operation are not available, but the method undoubtedly consists of shallow-pan fermentation of sucrose by *Aspergillus niger*, the process being completed in probably less than nine days. The mass of patent literature concerning this fermentation reveals little of actual plant operation.

The most recent work on the shallow-pan process to be published is that of Doelger and Prescott (1934). This method depends on the acidification of the medium to pH 1·6–2·2 with HCl, to suppress contaminating yeasts and bacteria. An unusual method of citric fermentation was advocated by Cahn (1935) in which a rapid formation of acid was obtained by using a relatively larger surface of mycelium. This was secured by growing the mould through a mass of bagasse or beet pulp impregnated with various sugar solutions.

The production of calcium gluconate by mould fermentation has been studied extensively by a number of workers in the U. S. Department of Agriculture. In general, it may be produced by the shallow-pan method or by using submerged mould growths under increased air pressure. The latter is one of the most interesting developments of fermentation technique in recent years. The method is described by Herrick, Hellbach, and May (1935), and has resulted in the conversion of a number of surface fermentation processes, with their attendant drawbacks, to a type of rapid deep fermentation.

The object of the work described in this paper was to develop a method of producing citric acid and calcium gluconate which might be practicable in this country under existing circumstances. Shortages of suitable carbohydrates had to be considered as well as scarcity of nitrates and phosphates for nutrient media. In working on this method the equipment which might be required for large-scale operation had also to be kept in mind.

Methods.

Fermentations, whether on liquid or absorbent media were carried out in tinned dishes, 12″ × 10″ × 2½″, which were double painted with cellulose lacquer, and covered by glass plates. When the fermentations were carried out on absorbent media, the mass was pressed out in a tincture press, and the pressed cake moistened and re-pressed twice. Citric acid was estimated by precipitation as calcium citrate, which was dried and weighed. Calcium gluconate was estimated by precipitation of the calcium as oxalate, which was washed and titrated by permanganate.

Experimental.

Doelger and Prescott's (1934) method was investigated by one of us (O.R.), and results in substantial agreement with those published were obtained. Layers of sugar solution and nutrients 2·5 cm. deep, acidified to pH 1·8–2·0, were inoculated with *Aspergillus niger*. Mats started to form on the third day and increased until the eighth or ninth day. Provided sporing did not take place before the fifth day, yields of 33–35 per cent. citric acid in nine days at 28° C. were obtained. The strength of the acid varied between 6 per cent. and 8 per cent. The mats could be used a second or third time to produce more citric acid if fresh sugar solution equal in strength (12 or 15 per cent.) to the original culture solution was run in. Oxalic acid was seldom produced by the first fermentation of a mat, but the second and third fermentations often produced small quantities. The acid yield and concentration of these fermentations was generally the same as in the primary fermentation. These laboratory experiments were reproduced with a total area of 40 sq. feet of mycelium, and similar results as to yield and strength were obtained. The main objections to the operation of this method under present circumstances in this country include: (1) the difficulty of securing metal pans; (2) the high cost and scarcity of the main raw material, sugar; (3) the length of the fermentation period; and (4) the liability of the *Aspergillus* mats to infection by a parasitic *Penicillium*. When this occurs the yield of citric acid is very low.

Cahn's Process.

An original method was described by Cahn (1935) in which molasses and other sugar-containing solutions are absorbed by beet pulp or bagasse, inoculated, and fermented for two to three days. The acid is recovered by diffusion, on the counter-current principle, from the chopped fermented mass. This method seemed to offer distinct advantages, and was accordingly investigated. Cahn, who used cane molasses, stated that beet molasses was toxic to his strain of *Aspergillus*. Bagasse not being available in Ireland at present, beet pulp impregnated with nutrient solution of the Doelger and Prescott (1935) formula was used. It was found very troublesome to get the dry beet

pulp saturated with solution, boiling for 30 min. being necessary. The growing mycelium then penetrated the soft tissues of the impregnated pulp, and the mass at the end of 48 or 60 hours had the consistency of soft cheese. The recovery of acid from this was difficult, nor could the high yields reported by Cahn be obtained. He may have used a strain of *Aspergillus* better adapted to the conditions of his method than those available to us.

THE SPHAGNUM METHOD.

Consideration of these results led to the trial of other absorbents available here. One of the most obvious of these is sphagnum moss, which is abundant and, by virtue of its peculiar leaf structure, an excellent retainer of moisture. Furthermore, it absorbs liquids by capillarity and not by imbibition, as in the case of beet pulp, and when used in the right proportion has a "channelled" texture through which air can penetrate to the germinating spores and mycelium. Finally, the fermented solution need not be diffused, but can be pressed out. The sphagnum from the bog is first pressed to remove surplus water, and then dried at a temperature of 80° C. to 100° C. This drying is important, as germination of the *Aspergillus* is retarded and yields are low when it is omitted. The moss is used in the proportion of 50 g. of air-dry sphagnum to 500–600 c.c. of solution, which is added hot. When cool, the mass is heavily inoculated with *Aspergillus* spores, and well mixed to distribute them. The spores germinate in 5–12 hours, and fermentation proceeds for 48 to 72 hours. The mycelium generally spores on the third day, but should be pressed out 48 hours after inoculation. The depth of saturated moss is between 1 and 2 inches, and the temperature of the fermentation chamber should be 25° C. If the depth is greater than 2 inches or the external temperature higher than 25° C., over-heating up to 42° C. tends to occur within the sphagnum. When this happens the yields are always low.

Other absorbents tested were apple pomace, malt combings, and softwood sawdust. The first two were unsatisfactory, their low water absorbing powers necessitating the use of relatively large quantities. In the case of apple pomace, the mycelium tended to grow on the surface of the mass. With malt combings the final condition resembled the cheese-like mass obtained when beet pulp was used; also yields of citric acid were very low. Sawdust used in the proportion of 120 g. per 600 c.c. of solution was quite satisfactory, giving yields comparable to those obtained on sphagnum.

Three different fermentable solutions were used in this work, the first being the Doelger and Prescott (1934) solution—

Sucrose	150	grams per litre acidified to pH 2·0 with HCl.
NH_4NO_3	2·3	
KH_2PO_4	1·0	
$MgSO_4$	0·25	

On a two-day fermentation the average yield of citric acid in three experiments was only 15 per cent. of the sugar taken, and a quantity of oxalic acid was formed. Allowing the fermentation to proceed for 3 days did not improve the yield. When the sucrose concentration was raised to 20 per cent. the yield in two days was increased to 18 per cent., and the tendency to form oxalic acid was markedly reduced. Different strains of *Aspergillus* were then tried, but did not effect any improvement. The only apparent advantages in using this formula with sphagnum, instead of in shallow pans, were the freedom from infection and the shorter fermentation time. These advantages were offset by the low yields of acid.

Attention was then turned to molasses, which is a cheap source of sucrose and contains sufficient minerals and nitrogenous materials for the growth of *Aspergillus.* While Cahn (1935) has reported beet molasses as being toxic to his strain of *Aspergillus,* it was felt that it might be possible to overcome this toxicity. Chattergee (1942) describes a shallow-pan method of fermenting molasses—presumably cane molasses—below pH 3·5, using aluminium pans. Our first experiments with beet molasses at different concentrations up to 40 per cent., and at pH 2·0, 3·0, and 5·0, in liquid form in shallow pans were always unsuccessful. Spore germination and growth of the mats was very slow, while infection was heavy. Small amounts of citric acid were obtained by growing the mould in conical flasks containing 200 c.c. of sterile 25 per cent. molasses, yielding 2·91 g. of calcium citrate in 10 days, and 4·62 g. in 14 days. However, when sphagnum or sawdust was impregnated with dilute beet molasses, rapid growth took place, in marked contrast to the poor growth on liquid molasses media. Acidification to pH 2·0 practically stopped growth, consequently the diluted molasses was used at its natural pH (7·2).

The fermentation medium in these experiments was 200 g. molasses brought to boiling point with 450 c.c. water, poured on to 50 g. dry sphagnum, inoculated when cool and fermented for two days. The pressings were neutralised with calcium carbonate, filtered, and boiled to precipitate calcium citrate. The yields of these experiments were 10–12 g. of citrate per 200 g. of molasses. Adding NH_4NO_3 and KH_2PO_4, together or separately, to the molasses mashes did not improve the yields. Though the results were poor, these experiments did show that beet molasses could be fermented in two days to produce some citric acid.

The next change in the method was influenced by the following considerations:—

1. Analyses figures of Irish beet molasses show a very high ratio of sodium and potassium ions to calcium, and it was thought that increasing the calcium content of our molasses mashes might antagonise the possibly deleterious effects of this high concentration of sodium and potassium ions.

2. Moyer *et al.* (1937) have described a method of obtaining calcium gluconate by fermenting sugar solutions containing calcium carbonate

with *A. niger* in rotating drums under air pressure. This method gave high yields with a short fermentation period. In view of the short fermentation period with sphagnum molasses for citric acid it was thought that calcium gluconate might also be produced by the sphagnum method without the necessity for the elaborate pressure drums of Moyer.

3. The presence of calcium carbonate in the fermentation mixture has been claimed by some workers (Wehmer, 1893, Chrzaszcz and Peyros 1935) to increase the yield of citric acid, but in the shallow-pan method its use naturally favours contamination.

A preliminary experiment using a sugar solution of the following formula was set up:—

Sucrose	100 g.	Sphagnum moss	50 g.
NH_4NO_3	1·5 g.	$CaCO_3$	25 g.
KH_2PO_4	0·15 g.	Water	to 600 c.c.
$MgSO_4 7H_2O$	0·12 g.		

Growth of the *Aspergillus* was quite vigorous, and after three days the mass was pressed. The pressings were filtered from unused carbonate, and, on boiling, a precipitate of 9·65 g. of calcium citrate was obtained. When the citrate was filtered off, the filtrate was evaporated to a syrup, and, on standing, a copious precipitate of calcium gluconate was obtained.

As a result of some 70 experiments using molasses the following formula was found to give the most satisfactory yields:—

Molasses	200 g.
Sphagnum	50 g.
Calcium carbonate	40 g.
Water	to 600 c.c.

Growth on this medium at a temperature of 25° C. was vigorous, and when the fermentations were pressed, filtered, and boiled, copious precipitates of calcium citrate were obtained. The yields varied somewhat with individual pans, but in most cases ranged between 25 g. and 30 g. of calcium citrate per pan in two days. Increasing the time to three days did not improve the yield of citrate, neither did the addition of traces of $ZnSO_4$ and $Fe_2(SO_4)_3$. Increasing the molasses to 300 g. per pan gave relatively lower yields. A higher temperature than 25° C. always resulted in overheating of the fermenting moss; after the spores had germinated, the internal temperature would rise to 42° C. some 24 hours after inoculation. Overheated pans always gave negligible quantities of citrate. Using the impregnated sphagnum at a greater depth than 1·5 inches in an attempt to increase the output per pan also resulted in overheating, even at an air temperature of 25° C. Whether this heating was due to respiration of the mycelium or thermophilic bacteria was not investigated. The calcium citrate obtained from molasses was a pale-

grey powder, which gave a dilute solution of light-brown citric acid on treatment with sulphuric acid. This dilute citric acid was decolorised with charcoal, and on concentration crystallised readily to a white or very pale-yellow product.

The fermented molasses from which the citrate had been filtered was evaporated down to 200 c.c., and on standing calcium gluconate crystallised out. This gluconate could be freed from molasses residues by pressing in cloth, or by washing with small amounts of water. This procedure resulted in a pale-brown product which could be further purified by treatment with charcoal and re-crystallising from water to give white calcium gluconate.

The yields of gluconate—which was produced simultaneously with the citrate—varied with individual pans, but in general lay within the range of 50 g. to 65 g. of calcium gluconate per 200 g. molasses in a two-day fermentation.

Re-examination of the three-day molasses-sphagnum fermentations without calcium carbonate showed that much less gluconate was produced, as might be expected. Without calcium carbonate, 200 g. molasses yielded only 20–25 g. of gluconate on neutralisation. Some workers cited by Prescott and Dunn (1940) have claimed that gluconic acid is a stage in the formation of citric acid. When sphagnum was impregnated with a solution of 100 g. calcium gluconate in 600 c.c. water, plus a little molasses to supply nutrients, the mycelium developed well, but no citrate was produced. In this method it seems that calcium gluconate will not ferment to citric acid.

The results of all these experiments may be summed up in the following table:—

Medium.	Method.	Time. Days.	Ca. Citrate. Grams.	Ca. Gluconate. Grams.
100 g. Sucrose + Nutrient salts (1)	Shallow Pan	9	48·3	0
100 g. Sucrose + Nutrient salts (1)	Sphagnum Moss	2	27·1	0
200 g. Molasses. Water to 600 c.c.	Sphagnum Moss or Sawdust	2–3	11	20–25
200 g. Molasses. 40 g. $CaCO_3$. Water to 600 c.c.	Sphagnum Moss or Sawdust	2	25–30	40–45

(1) NH_4NO_3 1·15 g., KH_2PO_4 0·5 g., $MgSO_4$ 0·12 g., Water 500 c.c.

Discussion.

In emergency conditions the sphagnum method offers many advantages over the shallow-pan process. Large areas of acid-proof metal pans and quantities of NH_4NO_3 and KH_2PO_4 are not required. Trays built of asbestos

slate screwed to wooden battens have given satisfactory yields in the laboratory. The rapid fermentation rate prevents the infection by other fungi, which was found to be very troublesome in the 9-day process. Bacterial contamination might be expected from the working conditions of the method, but it was never noticed; this may be due to the secretion of antibiotics by *A. niger*.

Although the yield of citric acid on the sugar in molasses is lower than in the shallow-pan method, this is offset by the cheapness of molasses, and the rapidity and certainty of the fermentation. The value of the calcium gluconate produced simultaneously must also be considered. The total yield of citric acid plus gluconate is in the neighbourhood of 60–65 per cent. of the sugar present in the molasses.

Other minor advantages of this method are: (*a*) that on a large scale it avoids the difficulties of handling large volumes of liquid at a shallow depth, and (*b*) the troublesome neutralisation of dilute citric acid with calcium carbonate takes place during the fermentation as the acid is formed.

The process differs from Cahn's method in several important respects. The fermentation products are pressed out, not diffused. Beet molasses, which Cahn found to be toxic, is rendered usable by the addition of calcium carbonate. Calcium gluconate is produced along with calcium citrate. Since the effect of calcium carbonate on the gluconic acid fermentation of *A. niger* is well known (Prescott-Dunn, 1940, p. 380), it is not surprising that the yield of gluconate is improved by its addition.

It is not generally considered necessary to add calcium carbonate in the citric acid fermentation by the pan method, as its use favours contamination. Nevertheless the earlier workers did use calcium carbonate to increase the yield of citric acid. According to May and Herrick (1930) the infections favoured by the presence of calcium carbonate during a nine-day fermentation caused the process to be abandoned. Recent workers, as quoted earlier in this paper, have mainly tended to use sucrose media of very low pH with the surface method. Although a good deal of the conflicting data of various workers on citric acid may be due to the idiosyncrasies of different strains of *A. niger*, there is no doubt that the addition of calcium carbonate to the molasses solutions in this method nearly trebled the yield of calcium citrate. Further experiments using other strains of *Aspergillus* might result in increasing the ratio of citrate to gluconate.

SUMMARY.

1. The shallow-pan process and Cahn's method have been investigated. For various reasons neither method is practicable under present conditions.
2. A method of fermenting beet molasses with *Aspergillus niger* on a base of sphagnum moss or sawdust is described.
3. The fermentation time is two days, calcium citrate and calcium gluconate being produced simultaneously.

We wish to record our thanks to Professor Doyle for his continued interest in the progress of this work, and to the Research Department of the Irish Sugar Co., Ltd., who kindly supplied us with data on beet molasses. The process described in this paper has been allocated a Provisional Patent 325/43.

REFERENCES.

1. Cahn, F. J.—*Ind. Eng. Chem.*, **27**: 201 (1935).
2. Chattergee, N. P.—*J. Indian Chem. Soc.*, Ind. and News Ed., **5** (1942).
3. Chrzaszcz, T., and E. Peyros.—*Biochem. Zeit.*, **280**: 325 (1935).
4. Doelger, W. P., and S. C. Prescott.—*Ind. Eng. Chem.*, **26**: 1142 (1934).
5. Herrick, H. T., R. Hellbach, and O. E. May.—*Ind. Eng. Chem.*, **27**: 681 (1935).
6. May, O. E., and H. T. Herrick.—*Ind. Eng. Chem.*, **22**: 1172 (1930).
7. Moyer, A. J., P. A. Wells, J. J. Stubbs, H. T. Herrick, and O. E. May.—*Ind. Eng. Chem.*, **29**: 777 (1937).
8. Prescott, S. C., and C. G. Dunn.—"Industrial Microbiology," New York, 1940 (McGraw-Hill Book Company Inc.).
9. Wehmer, C.—*Compt. Rend.*, **117**: 332 (1893).
10. Wells, P., and H. T. Herrick.—*Ind. Eng. Chem.*, **30**: 255 (1938.)

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